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Development and evaluation of an in vitro radiochemical assay for juvenile hormone biosynthesis in the black blowfly, Phormia regina (Meigen).

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DEVELOPMENT AND EVALUATION OF AN *IN VITRO* RADIOCHEMICAL ASSAY FOR JUVENILE HORMONE BIOSYNTHESIS IN THE BLACK BLOWFLY, *PHORMIA REGINA* (MEIGEN)

A Thesis Presented

By

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DEDICATION

To my parents, Ching-Fen and Tien-Shun Liu, whose love and encouragement through these years has made any of this possible.

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LITERATURE REVIEW

The Black Blowfly, Phormia regina (Meigen) (Diptera: Calliphoridae)- an Experimental Animal

The anautogenous blowfly, P. regina, was chosen as the experimental animal for three reasons:

1. It is easily reared.
2. Its réproductive-endocrine relationships had already been investigated.
3. Its reproductive cycle is easy to control since the liver meal triggers juvenile hormone (JH) production which leads to vitellogenesis. Thus, for the purpose of this study, corpora allata (CA) activity can be controlled by regulating the liver supply.

Nutritional Requirement and Ovarian Development

It is known that nutrition plays an important role in egg maturation processes in many insects (Wilkens, 1968). A protein diet usually is required for egg maturation. Engelmann (1970), in his review, indicated that lack of required food could affect reproduction in either/or both of two ways:

1. There are insufficient reserves to synthesize proteinaceous and lipid yolk for egg maturation,
2. The endocrine glands are suppressed in their hormone

production due to a lack of reserves or a lack of some feeding stimulus.

Thus, two functions of dietary protein as providing building materials and triggering certain initial processes of oogenesis in P. regina have been suggested (Pappas and Fraenkel, 1977; Fraenkel and Hollowell, 1979).

The requirement for an exogenous source of protein has also been demonstrated for non-dipterous species. Mundall and Engelmann (1977) reported that in the reduviid, Triatoma protracta, a blood meal, as well as mating, provided the necessary stimulus for the initiation of vitellogenesis. Tobe and Chapman (1979) demonstrated that starvation suppressed the activity of the CA and resulted in a cessation of oocyte growth in Schistocerca americana gregaria.

Although autogenous dipterous insects can mature their first batch of eggs without an adult protein meal, protein deprivation in adult autogenous fleshflies did reduce fecundity (Denlinger, 1971; Chadha and Denlinger, 1976). Lea (1964) found that altering the quality and quantity of the larval diet and withholding nutrients from newly emerged females of Aedes taeniorhynchus also reduced the expression of autogeny.

In anautogenous dipterous insects, vitellogenesis requires the consumption of proteinaceous food. In the case of the housefly, Musca domestica L., most females require additional RNA or its precursors in the diet so that more than one ovarian cycle would result (Morrison and Davies,1964). Bodnaryk and Morrison (1966) demonstrated, using M. domestica, that an adequate adult protein diet can initiate and retain ovarian development by elevating the content of one or more specific

hemolymph proteins. Sakurai (1977) concluded that JH titer, as well as nutrient supply are important factors for vitellogenesis in M. domestica. Wilkens (1968) and Denlinger (1971) found that females of the fleshfly, Sarcophaga bullata Parker, required dietary amino acids or proteins for yolk deposition. Tobe and Langley (1978) indicated the importance of blood meals to oocyte maturation for tsetse fly (Glossina sp.). Various other researchers demonstrated the requirement of protein meals for egg maturation in Lucilia sericata (Hobson, 1938; Williams et al., 1977) and Calliphora erythrocephala (Fraenkel, 1940; Strangways-Dixon, 1961). Feinsod and Spielman (1980) postulated that the quantity of nutrients stored by mosquitoes appeares to determine whether additional nutrients must be ingested before previtellogenic development commences and pointed out the possibility that many mosquitoes may indeed require two blood meals before maturing eggs.

Rasso and Fraenkel (1954) first reported that the adult blowfly, P. regina, required a protein diet for egg development. A sugar and water diet is insufficient for ovarian development. This has been confirmed by Orr (1964a, 1964b), Mjeni and Morrison (1973, 1975, 1976), Stoffolano (1974), Pappas and Fraenkel (1977, 1978), and Fraenkel and Hollowell (1979). Orr (1964a) stressed that ingesting sufficient protein is important for the activation of the CA. One function of the protein meal, as a trigger of JH action, has been suggested by Orr (1964a), Pappas and Fraenkel (1977, 1978), and Fraenkel and Hollowell (1979). Orr (1964b) and Mjeni and Morrison (1973, 1976) demonstrated that female specific proteins are synthesized by the fat body from dietary protein

and released into the hemolymph where they are finally removed by the ovaries for egg maturation. Mjeni and Morrison (1976) concluded that the protein meal in P. regina has a dual function:

1. It somehow activates the CA to synthesize and release JH which influences the development of follicles.
2. It also provides precursors for the synthesis of female specific proteins for egg development.

To date, no experimental evidence has demonstrated that the protein meal really activates the CA to produce JH; and,in fact no one has demonstrated that the CA of P. regina really produce JH.

The Role of Juvenile Hormone in Ovarian Development

Juvenile hormone plays important roles in oogenesis, particularly with respect to vitellogenesis in most insects. The CA is the defined source of JH in all insects studied to date (Engelmann, 1980). One exception is in the male adult cecropia moth where the CA produces JH acid and not JH (Peter et al., 1981). Reviews concerning the role of JH in ovarian development have been written by Engelmann (1968; 1970; 1980). Engelmann (1970) stated that CA are involved in promoting egg maturation for the majority of the insect species studied while in some species (e.g., Sarcophaga, etc.), the CA do not control egg maturation directly but rather do so indirectly through their effect on certain phases of metabolism (Wilkens, 1968; Chadha and Denlinger, 1976).

The involvement of JH in the control of ovarian development in different species studied appears to be different. It can regulate

previtellogenesis, promote vitellogenesis, or allow oocytes to mature beyond the vitellogenic phase.

Controlling vitellogenesis is the most common action of the CA found in many species including Locusta migratoria (Chen et al., 1976; 1979; Ferenz et al., 1981), Periplaneta americana (Bell, 1969), Leucophaea maderae (Engelmann, 1976), Eublaberus posticus (Bell and Barth, 1971), Nauphoeta cinerea (Wilhelm and Luscher, 1974; Buhlmann, 1976), Leptinotarsa decemlineata (de Loof and de Wilde, 1970), Danaus plexippus (Pan and Wyatt, 1971), Bombus terrestris (Roseler, 1977), Triatoma protracta (Mundall and Engelmann, 1977), Rhodnius prolixus (Abu-Hakima and Davey, 1977a; 1977b; Garcia et al., 1979), Drosophila sp. (Kambysellis and Heed, 1974; Handler and Postlethwait, 1978; Giorgi, 1979), and Acheta domesticus (Renucci and Strambi, 1983).

JH is needed for the previtellogenic growth in some species including Aedes aegypti (Gwadz and Spielman, 1973; Feinsod and Spielman, 1980; Flanagan and Hagedorn, 1977), Glossina flies (Tobe and Langley, 1978), Schistocerca gregaria (Tobe and Pratt, 1975a; Tobe and Chapman, 1979), and Diploptera punctata (Tobe and Stay, 1977). In M. domestica, JH was found to act, not only as an inducer of follicular growth, but also as a regulator of vitellogenesis (Adams, 1974; Sakurai, 1977).

In Manduca sexta, JH seems to allow oocytes to mature beyond the vitellogenic phase rather than being concerned with either vitellogenin synthesis or its uptake (Nijhout and Riddiford, 1974).

In P. regina, Orr (1964a) first demonstrated that the CA must be present for two days after liver feeding to allow for the initiation of

egg development. Mjeni and Morrison (1973) proved that part of the action of JH is to control the active synthesis of specific hemolymph proteins essential for egg development. Various researchers further elucidated that JH is required throughout the period of follicular development (Mjeni and Morrison, 1976; Pappas and Fraenkel, 1978; Fraenkel and Hollowell, 1979).

In Vitro Culture Techniques for the Corpora Allata

The development of *in vitro* CA culture techniques for studies of JH biosynthesis has been a prime research interest for many years. Roller and Dahm (1970) employed long-term organ cultures of isolated brain, corpora cardiaca, and corpora allata (Br-CC-CA) complexes from pupal *Hyalophora cecropia* (L.) to provide the first direct evidence that the CA is the site of synthesis and release of JH. They demonstrated that the CA, after long-term *in vitro* incubation, has the potential to release JH when implanted into larvae. This was later confirmed by other researchers (Judy *et al.*, 1973a; Pratt *et al.*, 1976). Roller and Dahm's discovery introduced a new approach to experimental investigations of the insect CA.

Subsequently, other researchers applied these long-term organ culture procedures to explore, *in vitro*, JH produced by isolated CA of various insects with the primary aim of synthesizing enough hormone for identification purposes (Judy *et al.*, 1973a; 1973b; 1975; Muller *et al.*, 1974; Jennings *et al.*, 1975). Schooley *et al.*, (1973) and Jennings *et al.*, (1975) also demonstrated the specific incorporation of simple

precursors, such as propionate, into the carbon skeleton of JH. Judy et al., (1973a; 1973b; 1975) and Jennings et al., (1975) showed that the S-methyl group of methionine was the source of the ester moiety of the produced JH. This finding was in agreement with that of Metzler et al., (1971) and was later confirmed by researchers using short-term culture techniques (Pratt and Tobe, 1974; Tobe and Pratt, 1974a; Weaver et al., 1980).

Unfortunately, the long-term organ culture techniques could not provide an indication of the synthetic activity of the freshly excised glands. The development of a short-term in vitro radiochemical assay by Pratt and Tobe (1974) therefore provided a convenient method for quantifying the rates of JH biosynthesis. Thus, the JH biosynthetic rate reflected the activities of isolated CA at the physiological stages prior to dissection. More specifically, this method permitted one to investigate the role of the CA in insect reproduction. Research employing a short-term, in vitro radiochemical assay has been performed on various species (Tobe and Pratt, 1975a; 1975b; Weaver et al., 1975; Tobe and Stay, 1977; Stay and Tobe, 1977; 1978; Lanzrein et al., 1978; Kramer, 1978; de Kort et al., 1981; Khan et al., 1982; Weaver et al., 1980; Roseler et al., 1980; Ferenz and Kaufner, 1981). Results of the above investigations indicated that JH biosynthesis is indeed related to the reproductive cycle.

Several factors concerning the development of in vitro culturing techniques are reviewed below.

Medium and Methionine Concentration

Grace's insect tissue culture medium with various modifications has been used for long-term in vitro organ culture of CA (Roller and Dahm, 1970; Judy et al., 1973a; 1973b; 1975; Schooley et al., 1973). In all above cases, 0.04 mM [methyl-^{14}C]methionine was used as the methyl donor for JH synthesis. Pratt and Tobe (1974) and Pratt and Weaver (1975) employed modified Ringer's solution containing 0.28 mM [methyl-^{14}C]methionine as the culture medium. Tissue-culture medium TC 199 was used in place of Ringer's solution for studies on Schistocerca gregaria (Tobe and Pratt, 1974a; 1974b; 1975a; 1975b; Pratt et al., 1975; Tobe and Saleuddin, 1977) with either 0.29 mM or 0.34 mM [methyl-^{14}C]methionine. Both of the above [methyl-^{14}C]methionine concentrations are within the range of 0.1-0.4 mM which encompasses the physiological concentration of this substrate in the hemolymph of S. gregaria (Tobe and Pratt, 1974a). Various investigations employing TC 199 containing [methyl-^{14}C]methionine at concentrations of 0.29 mM or 0.34 mM were later conducted on different insect species (Pratt and Weaver, 1975; Weaver et al., 1975; Pratt et al., 1976; Tobe and Stay, 1977; Feyereisen and Tobe, 1981; Lanzrein et al., 1978; Weaver et al., 1978).

The capacity of the CA from Tenebrio molitor to biosynthesize C_{16}JH in different vertebrate and invertebrate tissue culture media was compared by Weaver et al., (1980). High rates of JH biosynthesis were obtained at exogenous methionine concentrations ranging from 0.3-0.9 mM. Weaver et al., (1980) concluded that amino acids other than methionine, as well as co-factors and vitamins are without effect in short-term culture. More recently, modified MEM (Kramer, 1978; Khan et al., 1982;

Ferenz and Kaufner, 1981), Grace's medium (Roseler and Roseler, 1978; Roseler et al., 1980), specially formulated salt solution (Feldlaufer et al., 1982; Bowers et al., 1983), and Marks 19AB (Granger et al., 1982) were employed for research on various species. Weaver et al., (1980) strongly suggested that the tissue culture medium should be selected with great care and should approximate the hemolymph conditions, at least with respect to pH and the Na^+/K^+ ratio.

Medium pH

None of the researchers working on long-term CA culture techniques ever published the pH values of the culture medium used. A modified Ringer's solution with pH of 6.85 was employed in the original development of the short-term in vitro radiochemical assay (Pratt and Tobe, 1974). The effect of different H^+ ion concentrations on the rate of JH biosynthesis by the CA was first demonstrated using S. gregaria by Tobe and Pratt (1974a). They reported that the glands had a maximum synthetic activity at pH 8.0, but their activity was more reproducible in the physiological range pH 7.0-7.5. Pratt and his co-researchers subsequently adjusted culture medium pH to 7.2 for their investigations on S. gregaria and Periplaneta americana (Tobe and Pratt, 1975a; Pratt et al., 1975; 1976). Kramer (1978) demonstrated that the synthetic activity of the CA from Leptinotarsa decemlineata was constant between pH 6.0 and 7.5. The following researchers, therefore, employed a culture medium at pH 6.9 for the study on L. decemlineata (Khan et al., 1982). Weaver et al., (1980) investigated the influence of pH on the

rate of JH biosynthesis by CA from T. molitor and stressed the importance of adjusting the medium pH to a value consistent with the hemolymph.

Incubation duration

Roller and Dahm (1970) demonstrated that the CA of Hyalophora cecropia, kept for 6-7 days under in vitro conditions, still have the potential to release JH. Judy et al., (1973a) indicated that in M. sexta, more than half the glands maintained in vitro for up to 100 days still retained the ability to elicit a positive hormonal response using a bioassay. Pratt et al., (1976) reported that in P. americana, active glands aged in vitro for up to 40 hours do not have lower synthetic rates than contemporary glands aged in vivo.

Tobe and Pratt (1974a) demonstrated that significant quantities of labelled JH could be detected in the medium after a 10 minute incubation, that the rate of incorporation was constant for up to four hours and that the hormone was not stored in the CA. The phenomenon that the hormone was not stored to any appreciable extent within the glands was also observed by other researchers on other species (Tobe and Stay, 1977; Roseler and Roseler, 1978). Tobe and Pratt (1974b; 1975a) and Pratt et al., (1975) confirmed that the release rate of produced JH was strictly proportional to the synthetic rate of this hormone and the cultured glands released newly synthesized JH at constant rates for at least three hours. Similar linear relationships between release rates and synthetic rates of JH have been obtained using in vitro culture of CA from various species (Pratt et al., 1975; Tobe and Stay, 1977;

Kramer, 1978; de Kort et al., 1981; Khan et al., 1982; Roseler and Roseler, 1978). However, in some other cases, hormone synthetic rates were essentially constant for periods of 3-8 hours, and then diminished progressively (Lanzrein et al., 1978; Weaver et al., 1980; Ferenz and Kaufner, 1981).

Results of the above research supported the contention that the rate of hormone synthesis in vitro is similar to that in vivo. It is generally accepted that the short-term in vitro radiochemical assay, originally developed by Pratt and Tobe (1974), is a convenient method for measuring the spontaneous rate of JH synthesis by isolated CA.

C H A P T E R II

THE EFFECT OF SEX, AGE, AND FEEDING HISTORY ON HEMOLYMPH pH OF *PHORMIA REGINA* (MEIGEN) (DIPTERA: CALLIPHORIDAE)

Introduction

Hemolymph pH affects biochemical processes in all living organisms possessing a hemocoel because of its effect on enzyme activities. Information about insect hemolymph pH have been compiled by Buck (1953) and Altman (1961) with some supplementary information in the review by Wyatt (1961).

In dipterous insects, Glaser (1925), using colorimetric methods, reported that the hemolymph pH of adult *Musca domestica* is 7.2-7.6. Friedman (1959), employing a modified microglass electrode, reported that the hemolymph pH of *Phormia regina* (Meigen) was 6.96 ± 0.05.

For the purpose of developing an *in vitro* organ culture technique for *P. regina*, it was important to understand the *in vivo* hemolymph pH at different ages and under different dietary conditions. Thus, the objectives of this investigation were:

1. To examine hemolymph pH difference between sexes.
2. To observe within-sex changes in hemolymph pH several hours before and after feeding on liver.
3. To observe within-sex changes in hemolymph pH for five days following emergence.

Materials and Methods

Experimental animals

Phormia regina was maintained at 28-30°C, 60% R.H., and 24 hour photophase. Adult flies were reared in screened cages (61 x 61 x 61 cm), and supplied with sugar cubes, non-fat powdered milk, and water. Raw beef liver was added and served as both an additional protein source and the oviposition medium. Newly deposited eggs were collected and put into a 300-ml Pyrex® beaker with artificial diet (200 g powdered whole milk, 200 g Brewer's yeast, 8.5 g methyl parahydroxybenzoate, 40 g agar, and 3 l water) covered with a piece of gauze. Larvae hatched within 24 hours under the above rearing conditions. After 9-10 days, larvae were allowed to crawl from the media into a pan of sand where they pupated. The pupal stage lasted for 4-5 days. Emerging flies were collected over a 3-8 hour interval. In the first 2 days after emergence, flies were only supplied with a sugar and water diet. At 48 hours afer emergence, flies were allowed to feed on beef liver *ad libitum*. Only those flies feeding on liver were used as experimental flies. Hemolymph pH was measured every 24 hours for both sexes and for flies which were 1-5 days old and had fed on beef liver at 2 days post emergence.

Measurements of hemolymph pH

All pH determinations were made at ambient room temperature with a Fisher Accumet pH Meter Model 610A (accuracy being ± 0.02) equipped with a needle pH electrode (MI-408B, Microelectrodes Inc.) and a micro-reference electrode (MI-401, Microelectrodes Inc.). Electrodes were

held separately by two micromanipulators. Before measurements were performed, electrodes were cleaned thoroughly with a detergent in distilled water and were then standardized using a buffer solution (pH 7). During measurement, the fly was immobilized with Tackiwax®, and using a dissecting microscope, the mesoscutum was removed. Being careful not to pierce the alimentary canal, both electrodes were inserted into the thorax. Readings were taken within 1.5-2 minutes. Measurements of the hemolymph pH were completed within 3 minutes after opening the thorax. After each measurement, the electrodes were rinsed and checked with the standard buffer solution. For each treatment, at least 20 or more individual flies were measured. All the data were analyzed using one-way or two-way Analysis of Variance (ANOVA) at 5% level of significance.

Results

The mean hemolymph pH of a total of 284 flies was 7.08. Means of hemolymph pH for male (N = 142) and female (N = 142) flies were 7.07 and 7.09 respectively. Results of the two-way ANOVA showed no significant difference due to sex.

Results in Table 1 show the effect of diet on the hemolymph pH of 2-day-old *P. regina*. There were no significant differences in pH between sugar-water fed flies before or 3-4 hours after supplemented with beef liver.

Table 2 presents the hemolymph pH of female *P. regina* five days

Table 1. Hemolymph pH of 2-day-old, *P. regina* fed different diets ($N \geq 20$)

Sex	Sugar only ($\bar{X} \pm$ S.E.)	3-4 hr after feeding on liver ($\bar{X} \pm$ S.E.)
Female	7.09 ± 0.03a[1]	7.16 ± 0.05a
Male	7.10 ± 0.04a	7.14 ± 0.03a

[1]Means followed by the same letter are not significantly different (Student-Newman-Keuls range test $p < 0.05$).

Table 2. Hemolymph pH range of female *P. regina* for 5 days following emergence (N ≥ 20)

Days after emergence	Diet[1]	pH ($\bar{X}$ ± S.E.)
1	SW	7.10 ± 0.04ab[3]
2	SW+L[2]	7.16 ± 0.05b
3	SW	7.06 ± 0.03ab
4	SW	7.01 ± 0.04a
5	SW	7.10 ± 0.03ab

[1]SW, sugar-water diet; SW+L, sugar-water-liver diet.

[2]Hemolymph pH was measured on the 2-day-old flies 3-4 hrs after feeding on liver.

[3]Means followed by different letters are significantly different (Student-Newman-Keuls range test $p < 0.05$).

Table 3. Hemolymph pH range of male P. regina for 5 days following emergence (N ≥ 20)

Days after emergence	Diet[1]	pH ($\bar{X}$ ± S.E.)
1	SW	7.20 ± 0.03a[3]
2	SW	7.10 ± 0.04ab
2	SW+L[2]	7.14 ± 0.03a
3	SW	7.03 ± 0.03bc
4	SW	6.98 ± 0.03c
5	SW	6.99 ± 0.03c

[1]SW, sugar-water diet; SW+L, sugar-water-liver diet.

[2]Hemolymph pH was measured on the 2-day-old flies 3-4 hrs after feeding on liver.

[3]Means followed by different letters are significantly different (Student-Newman-Keuls range test $p < 0.05$).

following emergence. The hemolymph pH is constant in the first two days with only a sugar and water diet. There is, however, a trend toward a lower pH during the first 2 days after feeding on liver (i.e., 3 and 4 days post emergence). Following this, the pH increased to 7.10 ± 0.03. The statistical analysis, using Student-Newman-Keuls range test at 5% level, showed a significant difference between 0 day and 2 days after feeding on liver.

The hemolymph pH of male P. regina five days following emergence is shown in Table 3. Likewise, results of the Student-Newman-Keuls range test at the 5% level showed no significant difference during the first two days after emergence. However, there were changes toward a lower pH during the first 3 days after feeding on liver. A significant difference was found in the hemolymph between the first day following liver feeding and the following days.

Discussion and Conclusions

Results of this study indicated no correlations between hemolymph pH and sex or age in P. regina. A similar result was reported on some orthopterous insects by Bodine (1926). Glaser (1925) was also unable to demonstrate any correlation between hemolymph pH and age or between hemolymph pH and metamorphosis using Malacosoma americana, Bombyx mori and Musca demostica.

Babers (1941) and Levenbook (1950) demonstrated that the buffer capacities were U-shaped curves for southern armyworm Prodenia eridania and horse bot fly Gasterophilus intestinalis. I believe that the

buffering capacity of Phormia's hemolymph prevented drastic hemolymph pH fluctuation. Thus, hemolymph pH of P. regina was maintained at a physiological range of 6.98 to 7.20 during the 5 days post-emergence in both sexes.

While the pH of liver juice showed an active acidity of pH 4.8, it is improbable that the lowering of the hemolymph pH 1 to 2 days after liver feeding (Table 2 and 3) was a result caused solely by the liver juice. However, it is possible that the accumulation of acidic metabolites after ingesting liver resulted in the tendency toward acid changes observed a few days after feeding on beef liver in both sexes. Taylor et al., (1934), Ludwig (1934) and Craig and Clark (1938) indicated that there was a change toward a more acid condition as pupation approached. After pupation, the hemolymph pH increased and remained fairly constant throughout the pupal stage. They concluded that the more acid condition was due to the acid production of histolysis.

Various methods employed for the measurement of hemolymph pH have included (1) colorimetric method (Jameson and Atkins, 1921; Crozier, 1923-1924; Bodine, 1925; 1926; Glaser, 1925; Hoskins and Harrison, 1934; Marshall, 1939), (2) hydrogen electrode (Bishop, 1923; Bodine and Fink, 1925; Bodine, 1926), (3) quinhydrone electrode (Hoskins and Harrison, 1934; Babers, 1938), and (4) glass electrode (Craig and Clark, 1938; Boche and Buck, 1942; Levenbook, 1950; Wyatt et al, 1956; Friedman, 1959). The microglass electrode modified by Friedman (1959) had a capacity of measuring 1.3-1.5 ul hemolymph. The microelectrode and

vessel developed by Bodine and Fink (1925) required 15-20 ul fluid for each estimation while the microvessel for the glass electrode used by Taylor and Birnie (1933) could be filled by approximately 30 ul of fluid. The glass electrode employed by Craig and Clark (1938) was capable of handling fluid volumes of 50 ul or less. Other measuring methods usually required a large amount of hemolymph for a determination which ended in either diluting or pooling several samples together to provide enough hemolymph volume for a measurement.

Clegg and Evans (1961) reported a hemolymph volume of 6-7 ul for adult P. regina while Chen and Levenbook (1966) reported the hemolymph volume as 12.2 ul. Thus, using previously described methods, it was necessary to pool several samples together to provide enough hemolymph volume for reliable pH measurements. Compared with earlier in vitro measuring techniques, the method employed in this study could be performed in situ for individual flies within a short time period. This improved technique might avoid some of the problems which could happen during the preparation of hemolymph samples. Although some other factors might affect the accuracy of measurements, both individual and measuring variations might be minimized by taking a large number of hemolymoh samples.

One of the practical applications of insect hemolymph analysis is that of formulating media for in vitro studies with insect tissue (Wyatt, 1961). The present study on the hemolymph pH of P. regina was originally undertaken to devise incubation conditions for in vitro culture of Phormia regina corpora allata (CA).

CHAPTER III

DEVELOPMENT AND EVALUATION OF AN *IN VITRO* RADIOCHEMICAL ASSAY FOR JUVENILE HORMOME BIOSYNTHESIS IN THE BLACK BLOWFLY, *PHORMIA REGINA* (MEIGEN)

Introduction

Juvenile hormone (JH) functions as a gonadotropin which controls egg development in most adult female insects. It has been established that the corpora allata (CA) are the defined source of JH (Engelmann, 1980). One of the key factors governing the functional JH titer is JH biosynthesis and secretion by the CA. Therefore, developing an efficient and accurate monitoring method for CA activity is important for studies concerning the role of JH. Early research on CA activity could only be inferred from indirect observations involving allatectomy and hormone therapy, gland implantations and bioassay of extracts of hemolymph (Tobe and Pratt, 1975). A short-term *in vitro* radiochemical assay has been developed by Pratt and Tobe (1974) for the locust *Schistocerca gregaria*. This assay provides a valid method for quantitatively estimating the rate of biosynthesis of JH by the CA. Research concerning *in vitro* CA activity, as it is correlated to insect reproduction, has been investigated by using this method on various species from Orthoptera (Tobe and Pratt, 1975a; 1975b; Ferenz and Kaufner, 1981), Dictyoptera (Weaver *et al.*, 1975; Tobe and Stay, 1977; Stay and Tobe, 1977; 1978; Lanzrein *et al.*, 1978), Coleoptera (Kramer, 1978; de Kort *et al.*, 1981; Khan *et al.*, 1982; Weaver *et al.*, 1980), and

Hymenoptera (Roseler *et al.*, 1980). However, there is no report on the development of an *in vitro* CA culture for dipterous insects.

In *Phormia regina* (Meigen), as in most insects, the CA controls the synthesis of vitellogenic protein necessary for egg development (Orr, 1964; Mjeni and Morrison, 1973; 1975; 1976; Pappas and Fraenkel, 1978; Fraenkel and Hollowell, 1979). The adult blowfly, *P. regina*, requires a protein diet for egg development (Rasso and Fraenkel, 1954; Orr, 1964a; 1964b; Stoffolano, 1974; Mjeni and Morrison, 1975; Pappas and Fraenkel, 1977) which suggested that in *P. regina* the CA is somehow activated by the ingestion of dietary protein (Orr, 1964a; Mjeni and Morrison, 1976; Pappas and Fraenkel, 1977; Fraenkel and Hollowell, 1979). Currently, there is no information relating *in vitro* CA activity to the effect of diet in *P. regina*.

The objectives of this study were:

1. To develop an *in vitro* organ culture technique for the CA of the anautogenous black blowfly, *Phormia regina*.
2. To use the *in vitro* technique to evaluate (a) the effect of diet on CA activity and (b) the requirement of the CA for an exogenous methyl donor.
3. To use chromatographic analyses to evaluate and identify the biosynthetic product of the CA.

Materials and Methods

Animals and dissection

The anautogenous black blowfly, *Phormia regina* (Meigen), was

maintained under a 24 hour photophase, 28-30°C and R.H. 60%. Larvae were reared in 300 ml beakers (covered with pieces of gauze) on an artifical diet of 200 mg whole powdered milk, 200 mg Brewer's yeast, 8.5 mg methyl parahydroxybenzoate,40 mg agar and 3 l of tap water. Mature larvae were allowed to crawl from the media into a pan of sand where they pupated. Emerging flies were collected over a two or three hour interval. Flies were supplied with sugar cubes and water only during the first 48 hours after emergence, after which they were divided into two groups. Flies of the control group were maintained on a sugar and water diet, whereas those of the experimental group were supplemented with liver. Only females with an appetite for liver were transferred to another small cage where they were supplied with liver for a total of 24 hours.

All instruments were cleaned and sterilized with 70% ethyl alcohol before dissection was performed. Flies were immobilized, using CO_2 anesthetization, in a Petri dish with the head held forward by minuten pins. The specimen was then flooded with a blowfly saline (Chen and Friedman, 1975). By using this technique, the dorsal membrane of the neck was brought into clear view. Removing the cervical membrane and teasing away the longitudinal neck muscles and the two large lateral tracheal trunks reveals the roundish, unpaired corpus allatum (CA) and the light blue corpus cardiacum-hypocerebral ganglion (CC-HG) complex (Dethier, 1976). These are visible under a 50x binocular microscope. The unpaired CA and CC-HG complexes (CC-CA) were removed and transferred using a minuten pin into carbowax (Carbowax® 20M, Supelco, Inc.) coated

Kimble borosilicate glass culture tubes (6 x 50 mm) containing the culture medium, two complexes per culture.

The ovarian development of the dissected flies was examined under a 100x compound microscope. The method used to stage the ovaries was that of Adams and Mulla (1967).

In vitro culture and extraction

Carbowax coated tubes (inner surfaces were exposed to 5% carbowax for several hours; extra wax was rinsed with distilled water three times before the tubes were dried at 100°C) were used throughout all the experiments to reduce the possible binding of biosynthesized JH to glass (Giese et al., 1977). Two freshly-isolated CC-CA complexes were incubated in 50 ul of modified Eagle's minimum essential medium (MEM) with Hanks' salts (GIBCO Lab.) at pH 7.0 with 1 uCi [methyl-^{3}H]methionine (specific activity 80 Ci/mmol; New England Nuclear (N.E.N.), Boston MA) for each incubation. The medium was filter sterilized with a nylon-66 filter (0.2 um pore size, 47 mm-diameter, Rainin) and frozen. When needed, the medium was thawed and refrigerated. Incubation was carried out in the dark at 26°C with 200 rpm shaking (junior orbit shaker, Lab-Line Instrument, Inc.) for four hours unless otherwise specified. Incubation was terminated and cultured medium was extracted three times with 100 ul iso-octane (2,2,4-trimethylpentane) per extraction. For each extraction, the medium and iso-octane were thoroughly mixed to form an emulsion by vortexing and sonication. This mixing procedure was repeated three times. Such emulsified medium/iso-octane mixtures were centrifuged at 10,000 rpm for

10 minutes and the upper organic phase was carefully removed from the aqueous phase with a capillary pipette. Pooled organic phases from three extractions were evaporated to dryness in culture tubes (6 x 50 mm) under an air stream.

The incorporation rates of [^{3}H]methionine were quantified in a Beckman LS-150 liquid scintillation spectrometer with counting efficiency of 30.9%. Dried extract was reconstituted with 20 ul of distilled water and was added with 500 ul scintillation fluid (Aquasol-2) (N.E.N.). The sample tube containing 520 ul scintillation fluid and water mixture was placed in a 7 ml tube and both were put into a 20 ml scintillation counting vial to make a three-layered-counting vial system. Incubations without CA served as extraction controls for each experiment. Extractions of controls showed that only a small amount (0.016%) of added radioactivity was transferred to the organic phase. Radioactivity of the extraction control was considered background and substracted from that of experimental treatments. The rates of *in vitro* methionine incorporation by cultured CC-CA complexes were calculated and are presented as pmol x gland^{-1} x hr^{-1}. Calculations were based on the following formula:

$$\frac{(R - B) \times SF}{(2.22 \times 10^6) \times E \times A \times S \times N \times T} = \text{pmol} \times \text{gland}^{-1} \times \text{hr}^{-1}$$

R = Radioactivity of experimental culture (cpm)
B = Background radioactivity (cpm)
SF = Scale factor = 10^3
E = Counting efficiency (cpm/dpm)
A = [^{3}H-methionine]/[cold methionine]
S = Specific activity (80 Ci/mmol)

N = Number of glands cultured
T = Incubation duration (hr)
2.22×10^6 dpm = 1 uCi

Different [^{3}H]JH III concentrations were prepared by incorporating 0.115, 1.146, and 11.460 pmol [^{3}H]JH III (specific activity 11.0 Ci/mmol, N.E.N.) into 50 ul of culture medium and extracted as previously described in order to determine the extraction efficiency of [^{3}H]JH III using iso-octane. Experiments were conducted to determine whether glands and culture medium should be extracted together or separately. Glands were transferred into 50 ul fresh non-radioactive medium after incubation terminated, and both incubated medium and glands with fresh medium were extracted as previously described.

L-methionine concentration. To determine the optimum L-methionine concentration of culture medium affecting the rates of *in vitro* incorporation of [^{3}H]methionine by isolated CC-CA, different L-methionine concentrations of 0.00025 mM, 0.025 mM, 0.038 mM, 0.05 mM, 0.063 mM, 0.075 mM, 0.088 mM, and 0.1 mM were tested. Each methionine concentration was achieved by incorporating 1 uCi [^{3}H]methionine (specific activity 80 Ci/mmol, N.E.N.) to a specific amount of cold L-methionine (GIBCO Lab.) into 50 ul culture medium. Incubation was conducted under conditions previously described. Differences were tested using Student-Newman-Keuls range test at the 5% level.

Incubation duration. Incubations of various durations, including 4, 8, 12, 16, 20, 24, and 48 hr, were conducted to investigate the effect of incubation duration on the rates of *in vitro* methionine

incorporation. Two freshly isolated CC-CA complexes from 3-day-old liver fed females were incubated in medium with a final methionine concentration of 0.05 mM, and other conditions previously described. Differences were tested using Student-Newman-Keuls range test at the 5% level.

Medium pH. As previously determined (Chapter II), the hemolymph pH of P. regina averaged 7.08. Different pH values of 6.5, 7.0, and 7.5 were examined to compare the effect of pH on the rates of in vitro methionine incorporation by CC-CA from 3-day-old liver fed females. Both medium and saline pH were adjusted with either 1 N NaOH or 1 N HCl in a decontamination hood to avoid any possible contamination by microorganisms. Two freshly isolated CC-CA complexes from 3-day-old liver fed females were cultured as previously described. Differences were also tested using Student-Newman-Keuls range test at the 5% level.

Dietary effects on the in vitro methionine incorporation by CC-CA

CC-CA from 3-day-old females either maintained with a sugar and water diet for three days or supplemented with liver at 48 hours were cultured to examine the effect of nutrition on the methionine incorporation by CC-CA. Two freshly isolated CC-CA complexes were cultured as previously described. The difference was tested using Student-Newman-Keuls range test at the 5% level.

Chemical confirmation of synthesized products

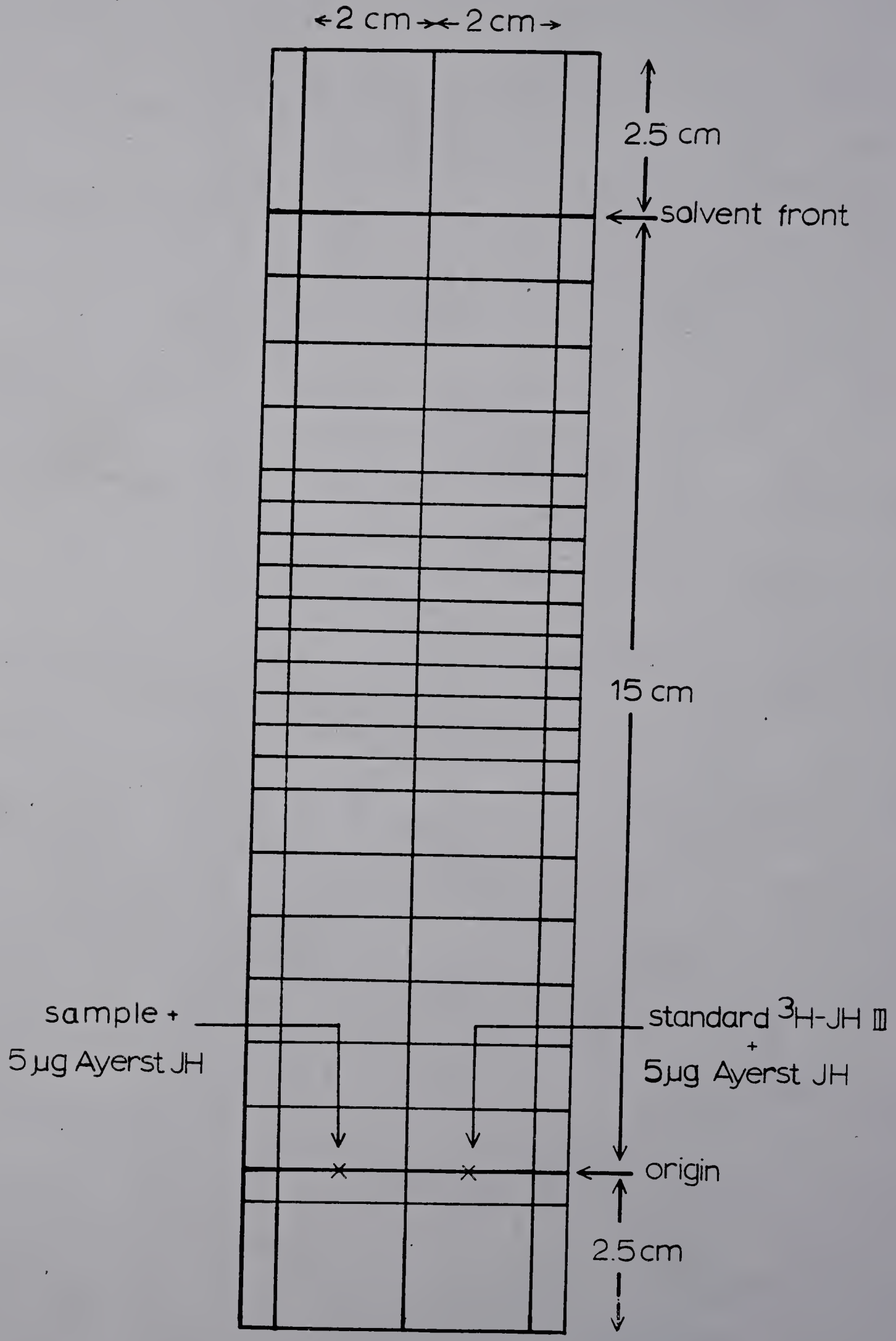
Qualitative analyses of in vitro biosynthesized products by CC-CA

of P. regina were conducted by using thin layer chromatography (TLC) with both normal phase and reverse phase, and reverse phase high pressure liquid chromatography (HPLC).

TLC. Reactivated silica gel TLC normal phase (with fluorescent indicator 13181, Eastman) and SiC-18 reverse phase (with fluorescent indicator 7013-1, VWR Scientific Inc.) plates were used for separation and identification of chemicals in the organic phases. For TLC normal phase, plates were cut into 5 x 20 cm strips. Each strip was marked at 2.5 cm from both the bottom and the top. These marks served as marks of origin and solvent front, respectively (Figure 1). Solvent was allowed to elute 15 cm when the strip was developed. There were always two applications spotted on each strip: one contained the organic extract of cultured sample while the other was the standard [^{3}H]JH III (specific activity 11.0 Ci/mmol, N.E.N.). Five ug of non-radiolabelled racemic JH I (Ayerst, Mckenna & Harrison Ltd., Montreal, Canada) was used as a carrier which not only made the spots visible under UV light, but also served as a reference for possible chemical identity.

Dried organic extract of the cultured sample was reconstituted with 30 ul iso-octane and was spotted at the origin where 5 ug JHI had already been spotted. The reference standard of 5 ug JHI and 0.0013 uCi (or 0.013 uCi) [^{3}H]JH III was then spotted on another origin of the same strip. The diameter of spots was never greater than 0.3 cm. TLC strips were developed with hexane : ethyl acetate at ratios of 1:1; 2:1; 1:4 v/v. As soon as the solvent reached the solvent front mark, the strip was removed from the developing chamber and set in a hood to let the solvent evaporate. The migrated components were examined and located

Figure 1. Diagram of the normal phase TLC strip (5 x 20 cm).

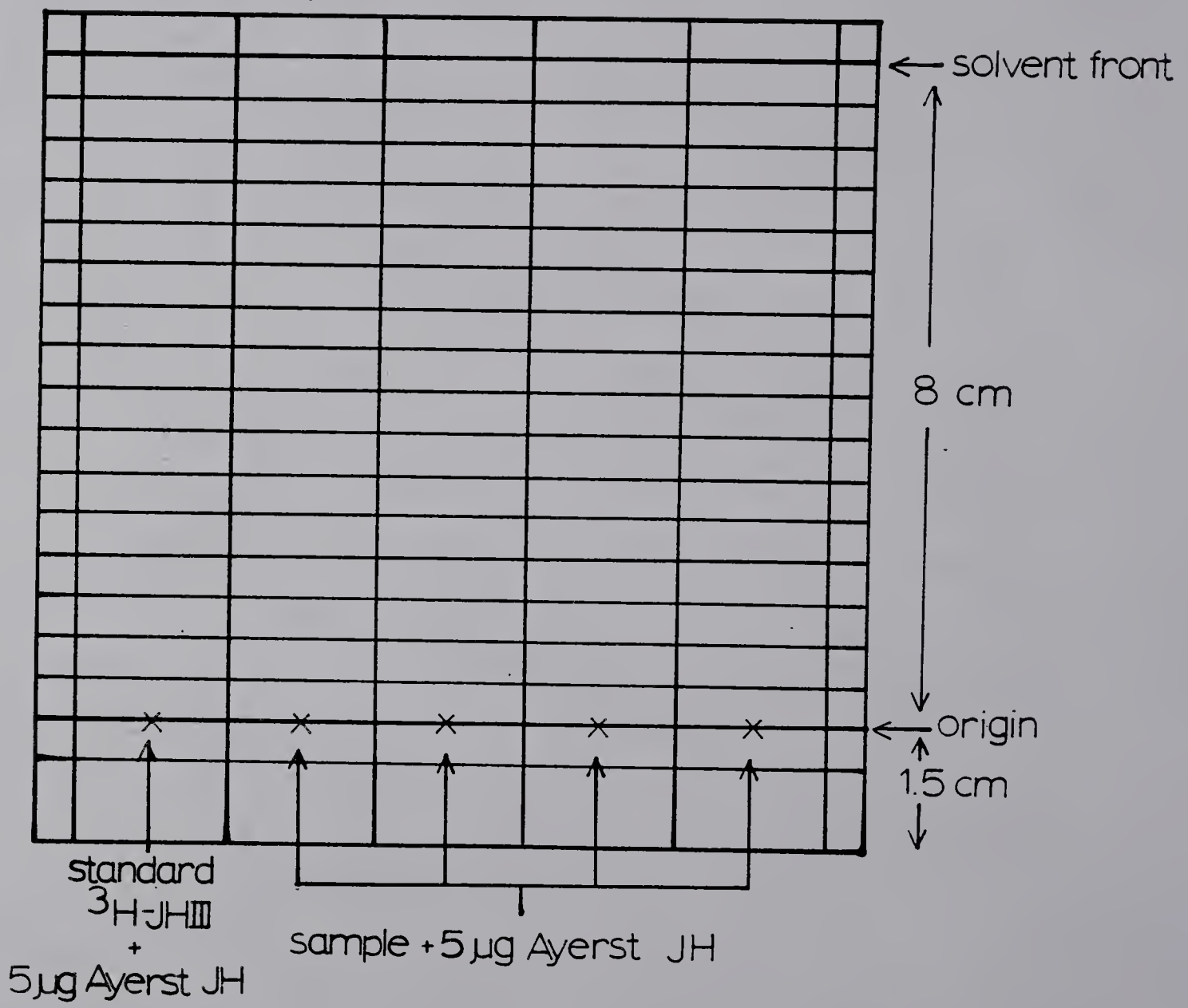

2 cm
2 cm
2.5 cm
solvent front
15 cm
sample +
5 µg Ayerst JH
standard ^{3}H-JH III
+
5µg Ayerst JH
origin
2.5 cm

under UV light. The whole strip was divided into either 2 x 1 cm or 2 x 0.5 cm fractions as shown in Figure 1. All the fractions were scraped off and resuspended in 20 ul distilled water and 500 ul scintillation fluid. The radioactivity was measured in a Beckman LS-150 liquid scintillation spectrometer. One 2 x 0.5 cm fraction of silica gel (without test spots) was added with 20 ul distilled water and 500 ul scintillation fluid and its radioactivity was counted as the control.

The size and arrangement of plates for TLC reverse phase were different from those for TLC normal phase. The plate size was 10 x 10 cm and divided into 5 strips (1.8 x 10 cm for each). The origin was 1.5 cm from the bottom while the mark for the solvent front was 0.5 cm from the top. The plate was developed with 70% methanol. After elution with solvent, the strip was divided into 1.8 x 0.5 cm fractions as shown in Figure 2. One blank fraction of the SiC-18 gel was also counted as a control.

HPLC. HPLC was performed with a Varian 5000 HPLC. Absorbance was recorded with a UV-VIS detector (Model 5000) at 220 nm. A C-18 reverse phase column (300 x 4 mm, Alltech) was used for separation. Methanol/water (80:20) served as eluent with a flow rate of 1.2 ml/min. Other parameters of the HPLC were: temperature 28°C, cut off frequency 0.02, gain 2, absorbance 0.02, and chart rate 1 cm/min. Purified JH I and JH III (Calbiochem-Behring Co.) were used as references. The column was always prewashed with 100% acetonitrile with 0.5 ml/min flow rate before sample separation. The dried organic extract of cultured sample was reconstituted with 30 ul 100% methanol (HPLC grade, Fisher Scientific Co.). Several samples were pooled together and reduced in

Figure 2. Diagram of the reverse phase TLC plate (10 x 10 cm).


solvent front
8 cm
origin
1.5 cm
standard
^{3}H-JHIII
+
5 µg Ayerst JH
sample + 5 µg Ayerst JH

volume. Only a 10 ul sample was injected each time. Fractions were collected with a Frac-100 fraction collector (Pharmacia). Fractions were collected at 30 second intervals. Each fraction was evaporated to dryness under an air stream and its radioactivity was measured.

Effect of an exogenous source of methionine on the methionine incorporation by CC-CA

CC-CA were cultured in MEM deprived of exogenous methionine to investigate the necessity of an exogenous methyl donor for *in vitro* methionine incorporation by the CC-CA of *P. regina*. Two CC-CA complexes were incubated in 50 ul MEM without methionine under conditions previously described. The cultured medium was extracted using procedures previously described. Samples were prepared for HPLC as previously described and were injected into HPLC using the same separation conditions described for previous experiments. The absorbance was monitored at 220 nm.

Results

Development of ovaries

Yolk first becomes evident in stage 4 in *P. regina*. Only 2.73% of the 146 flies fed liver failed to show signs of yolk while none of the 14 flies fed only a sugar and water diet showed signs of yolk deposition (Table 4). The results showed that 86.30% of the 146 flies examined 24 hours after liver feeding had reached stages 5, 6, and 7 with conspicuous yolk deposition.

Table 4. Ovarian development of 3-day-old female, P. regina, under different dietary conditions[1].

Ovarian development	+Liver on the 2nd day after emergence[2]	Sugar-water diet[3]
Stage	%	%
2	0.68	100
3	2.05	0
4	9.59	0
5	24.66	0
6	33.56	0
7	28.08	0
8	1.37	0

[1]CC-CA complexes of examined flies were used for in vitro culture.

[2]146 flies were examined.

[3]14 flies were examined.

Extraction efficiency and extraction sources

The extraction efficiencies (using 100 ul iso-octane each time to extract three separate times) were 91.2%, 91.8%, and 81.2% when [^{3}H]JH III concentrations were 0.115, 1.146, and 11.460 pmol/50 ul medium respectively. It indicated that the extraction efficiency was rather constant at lower [^{3}H]JH III concentration (0.115-1.146 pmol/50 ul medium). However, the extraction efficiency decreased when the [^{3}H]JH III concentration was increased another ten fold.

The data in Table 5 revealed that 97.8% of the radiolabelled products synthesized during a four hour incubation period could be recovered from the medium while only 2.2% was harvested from the glands. Based on this result, CC-CA complexes were extracted together with the medium in subsequent experiments.

L-methionine concentration

The influence of different L-methionine concentrations on the rate of *in vitro* ^{3}H-radioactivity incorporation by CC-CA from 3-day-old, liver-fed adult females is shown in Table 6. Although there were no significant differences among concentrations ranging from 0.025 mM to 0.063 mM, the maximal incorporation rate (2.09 ± 0.18 pmol x gland^{-1} x hr^{-1}) was obtained at a concentration of 0.05 mM. L-methionine concentration below 0.025 mM or above 0.075mM gave significantly ($p < 0.05$) lower rates of incorporation. Results also showed that only

Table 5. The presence of radioactive products from different extraction sources using an *in vitro* organ culture system for corpora allata from 3-day-old females of *P. regina*.

Extraction source	X ± S.E.[2] pmol x gland^{-1} x hr^{-1}
Medium	0.85 ± 0.11
Glands	0.02 ± 0.00

[1]All flies were fed with liver when 2 days old and dissected 24 hr after liver fed, two CC-CA complexes were incubated in 50 ul MEM containing 0.05 mM methionine, pH 7.0, at 26°C for 4 hrs.

[2]Each experiment replicated 3 times

Table 6. The effect of L-methionine concentration on the rate of *in vitro* incorporation by corpora allata from 3-day-old adult females of *P. regina*[1,2].

L-methionine conc.	Incorporation rate ($\bar{X}$ ± S.E.)[3]
mM	pmol x gland^{-1} x hr^{-1}
0.00025	0.0018 ± 0.0003c[4]
0.025	1.45 ± 0.18ab
0.038	1.48 ± 0.21ab
0.050	2.09 ± 0.18a
0.063	1.67 ± 0.32ab
0.075	0.92 ± 0.32b
0.088	0.31 ± 0.04c
0.100	0.08 ± 0.02c

[1]All flies were fed with liver when 2 days old.

[2]Two CC-CA complexes were incubated in 50 ul MEM (pH 7.0) at 26°C for 4 hrs.

[3]Each mean represents 6 replicates.

[4]Means followed by different letters are significantly different (Student-Newman-Keuls range test $p < 0.05$).

0.0018 ± 0.0003 pmol x gland^{-1} x hr^{-1} of incorporation can be detected when the methionine concentration was 0.00025 mM. The *in vitro* incorporation rate of ^{3}H-radioactivity had an 1,160-fold increase as methionine concentration increased from 0.00025 mM to 0.05 mM. However, the *in vitro* incorporation rate declined when the methionine concentration was greater than 0.05 mM.

Incubation duration

Statistical analysis using Student-Newman-Keuls range test showed a significant difference ($p < 0.05$) between incorporation rates for four hours duration and those incubated more than twelve hours (Table 7). However, there was no significant difference between a four hour and an eight hour incubation duration. The greatest incorporation rate (0.65 ± 0.14 pmol x gland^{-1} x hr^{-1}) was observed at four hours of incubation. As the incubation period increased to eight hours or longer, the incorporation rate declined and remained at the lower rate for up to 24 hours (Table 7).

Medium pH

The incorporation rate tended to increase when the medium pH changed from 6.5 to 7.5 (Table 8). Statistical analysis indicated a significant difference ($p < 0.05$) between pH 7.0 and pH 7.5, but no significant difference between pH 6.5 and pH 7.0. Based on the experiments conducted in Chapter II, the *in vivo* hemolymph pH value was 7.08 and, as a result, all futhur experiments were conducted at pH 7.0.

Table 7. The effect of incubation duration on the rate of *in vitro* incorporation of [^{3}H]methionine by corpora allata from 3-day-old adult females of *P. regina*[1,2].

Incubation duration	Incorporation rate ($\bar{X} \pm$ S.E.)[3]
hr	pmol x gland^{-1} x hr^{-1}
4	0.65 ± 0.14a[4]
8	0.51 ± 0.09ab
12	0.30 ± 0.03b
16	0.31 ± 0.08b
20	0.29 ± 0.04b
24	0.27 ± 0.05b
48	0.20 ± 0.02b

[1]All flies were fed with liver when 2 days old.

[2]Two CC-CA complexes were incubated in 50 ul MEM containing 0.05 mM methionine (pH 7.0) at 26°C for various times.

[3]Each mean represents 8 replicates.

[4]Means followed by different letters are significantly different (Student-Newman-Keuls range test $p < 0.05$).

Table 8. The effect of culture medium pH on the rate of *in vitro* incorporation of [^{3}H]methionine by corpora allata from 3-day-old adult females of *P. regina*[1,2].

pH	Incorporation rate ($\bar{X} \pm$ S.E.)[3] pmol x gland^{-1} x hr^{-1}
6.5	1.18 ± 0.37b[4]
7.0	2.10 ± 0.39b
7.5	3.56 ± 0.55a

[1]All flies were fed with liver when 2 days old.

[2]Two CC-CA complexes were incubated in 50 ul MEM containing 0.05 mM methionine at 26°C for 4 hrs.

[3]Each mean represents 6 replicates.

[4]Means followed by different letters are significantly different (Student-Newman-Keuls range test $p < 0.05$).

Dietary effects on the methionine incorporation by CC-CA

The data of Table 9 showed that the incorporation rate of the CC-CA from liver fed flies was 0.86 ± 0.09 pmol x gland^{-1} x hr^{-1}. For sugar and water fed flies the incorporation rate was only 0.07 ± 0.01 pmol x gland^{-1} x hr^{-1}, representing a 12-fold difference. Results of these two treatments were significantly different ($p < 0.05$) from each other.

TLC (normal phase) separation and identification

The distribution of radioactivity on TLC plates developed with different solvent systems are presented in Figures 3-10. The percentage of radioactivity is given only for those scrapings with a significant (eg > 3.9%) of total radioactivity. Each figure contains two parts: (A) the result from samples cultured in vitro for different incubation periods; and (B) the mobility of the reference standard which was eluted on the same TLC strip with (A). Presentation of data in this manner facilitates making comparisons and is the best way to present the material for discussing the relationship between the relative mobility of the cultured sample and the standard.

The radioactivity distribution of the extraction control (extract of incubation medium incubated without adding CC-CA) on TLC developed in hexane/ethyl acetate 2:1 is presented in Figure 3 and shows little radioactivity. As clearly shown in Figures 4-6, standard [^{3}H]JH III always co-migrated with Ayerst JH I (unlabelled marker) while cultured samples never co-migrated with Ayerst JH I and showed a 0.5 cm slower mobility when using hexane/ethyl acetate 1:1 as eluting solvent.

Table 9. The effect of diet on the rate of *in vitro* incorporation of [^{3}H]methionine by corpora allata from 3-day-old females of *P. regina* under different feeding conditions[1].

Dietary condition	Incorporation rate ($\bar{X} \pm$ S.E.)[2] pmol x gland^{-1} x hr^{-1}
Sugar-water (SW)	0.07 ± 0.01b[3]
SW + liver[4]	0.86 ± 0.09a

[1]Two CC-CA complexes were incubated in 50 ul MEM containing 0.05 mM methionine (pH 7.0) at 26°C for 4 hrs.

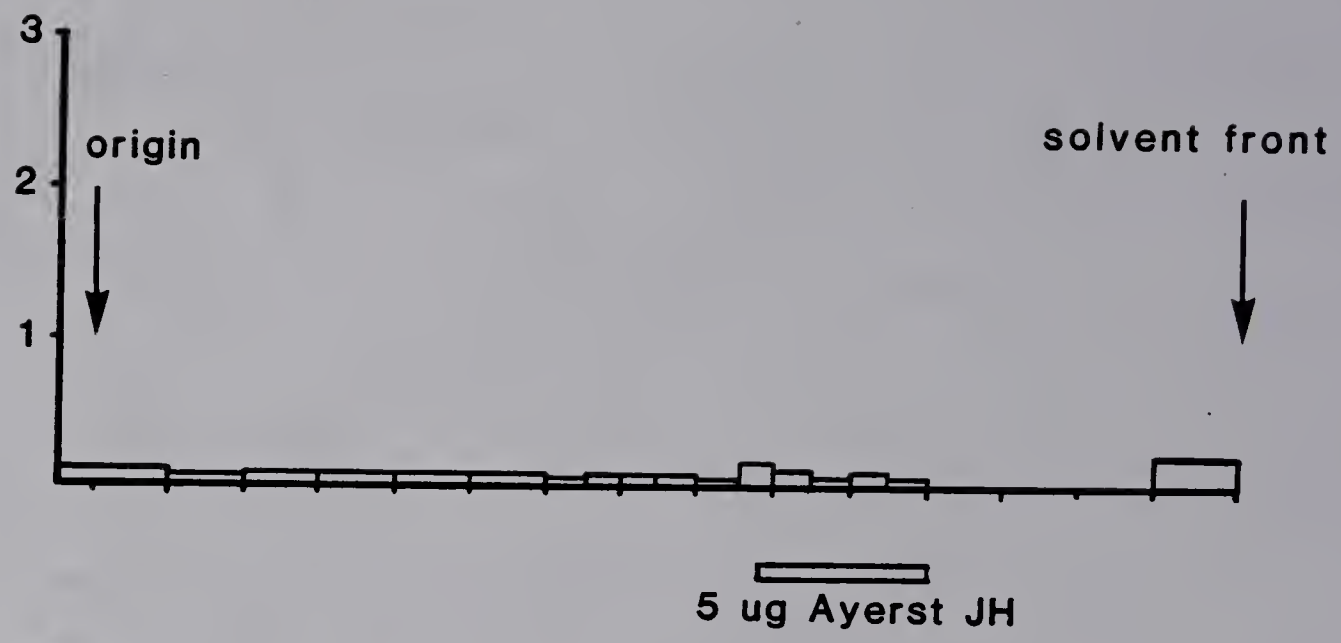
[2]Each mean represents 22 replicates.

[3]Means followed by different letters are significantly different ($p < 0.05$).

[4]Flies were fed liver for 24 hours starting on the 2nd day.

Figure 3. The distribution of radioactivity from the extraction control (A), and reference standard (B) on TLC normal phase developed in hexane/ethyl acetate 2:1.

A) Control



B) Reference standard

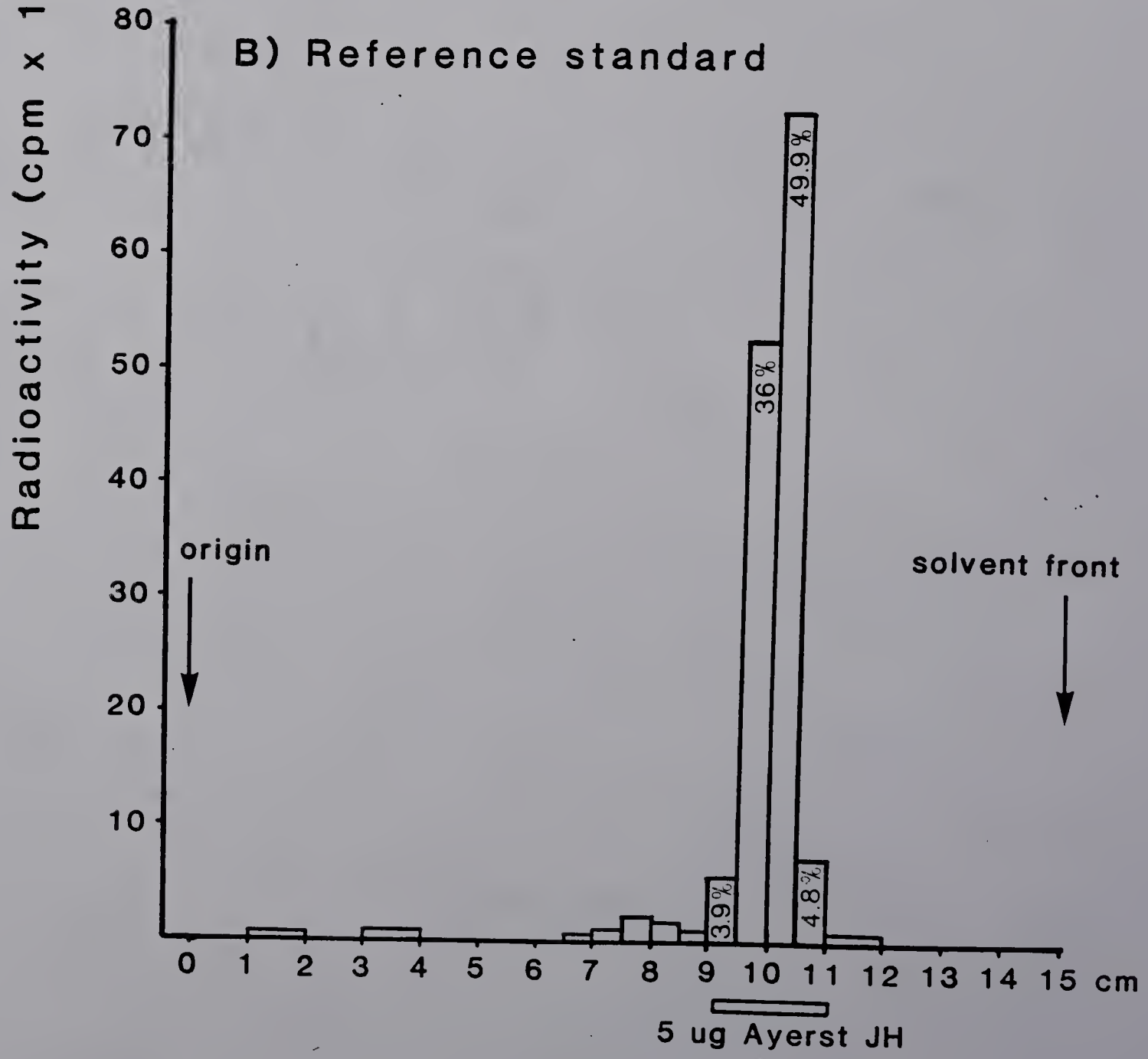


Figure 4. The distribution of radioactivity from the extract of CC-CA cultured for 4 hrs (A), and reference standard (B) on TLC normal phase developed in hexane/ethyl acetate 1:1.

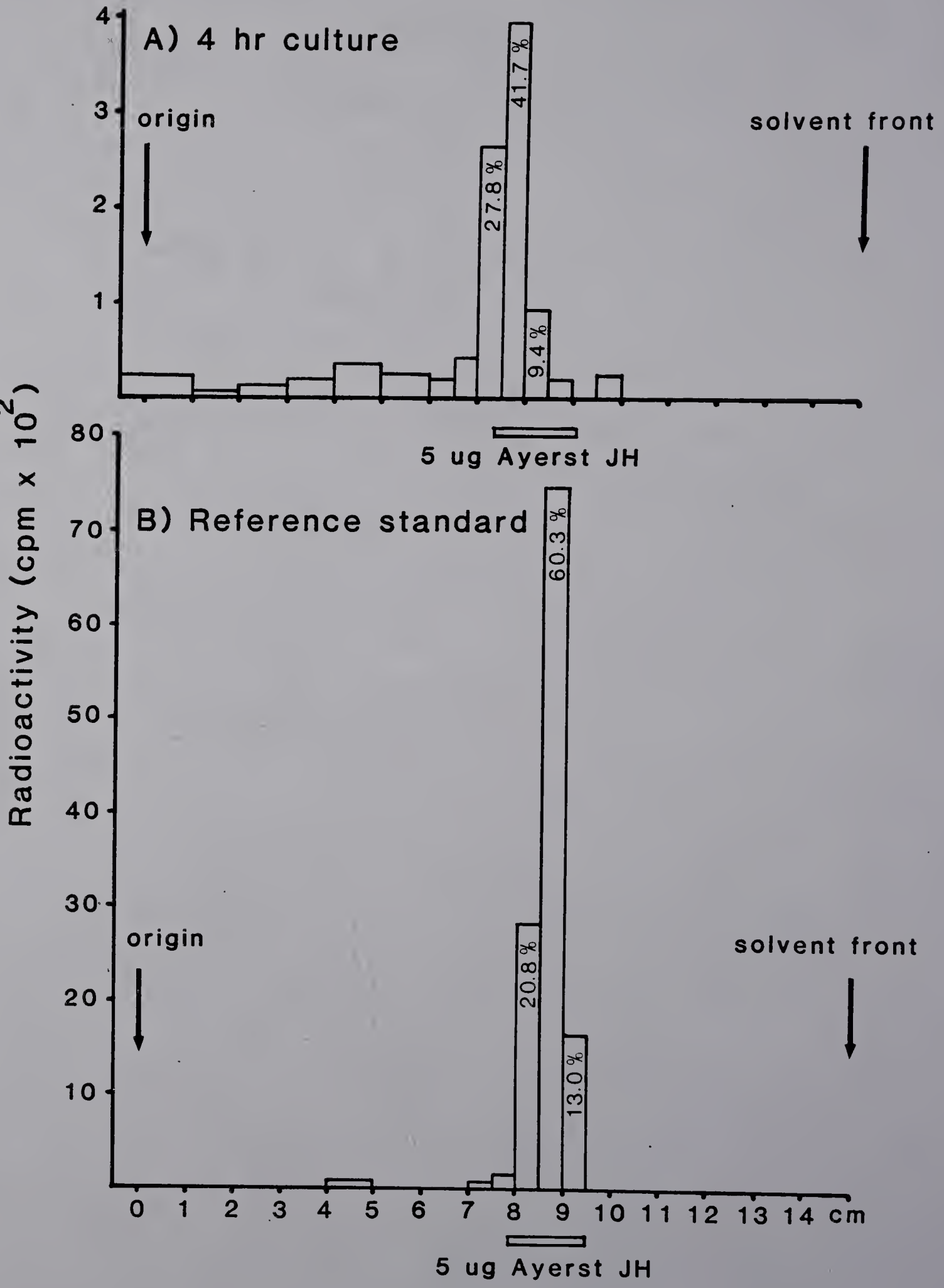
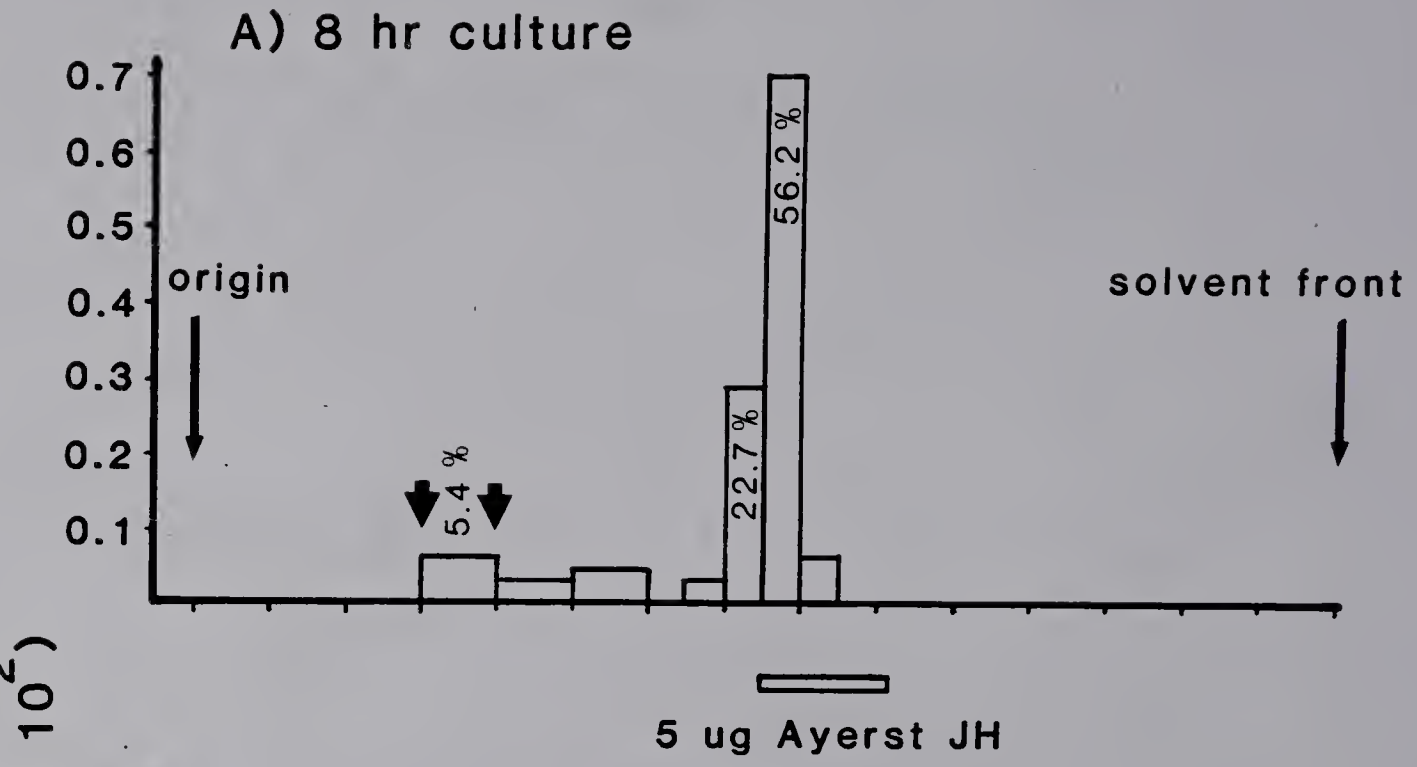

A) 4 hr culture
origin
solvent front
27.8 %
41.7 %
9.4 %
5 ug Ayerst JH
B) Reference standard
60.3 %
20.8 %
13.0 %
origin
solvent front
5 ug Ayerst JH
Radioactivity (cpm x 10²)
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 cm

Figure 5. The distribution of radioactivity from the extract of CC-CA cultured for 8 hrs (A), and reference standard (B) on TLC normal phase developed in hexane/ethyl acetate 1:1.


A) 8 hr culture
0.7
0.6
0.5
0.4
0.3
0.2
0.1
origin
solvent front
5.4 %
22.7 %
56.2 %
5 ug Ayerst JH

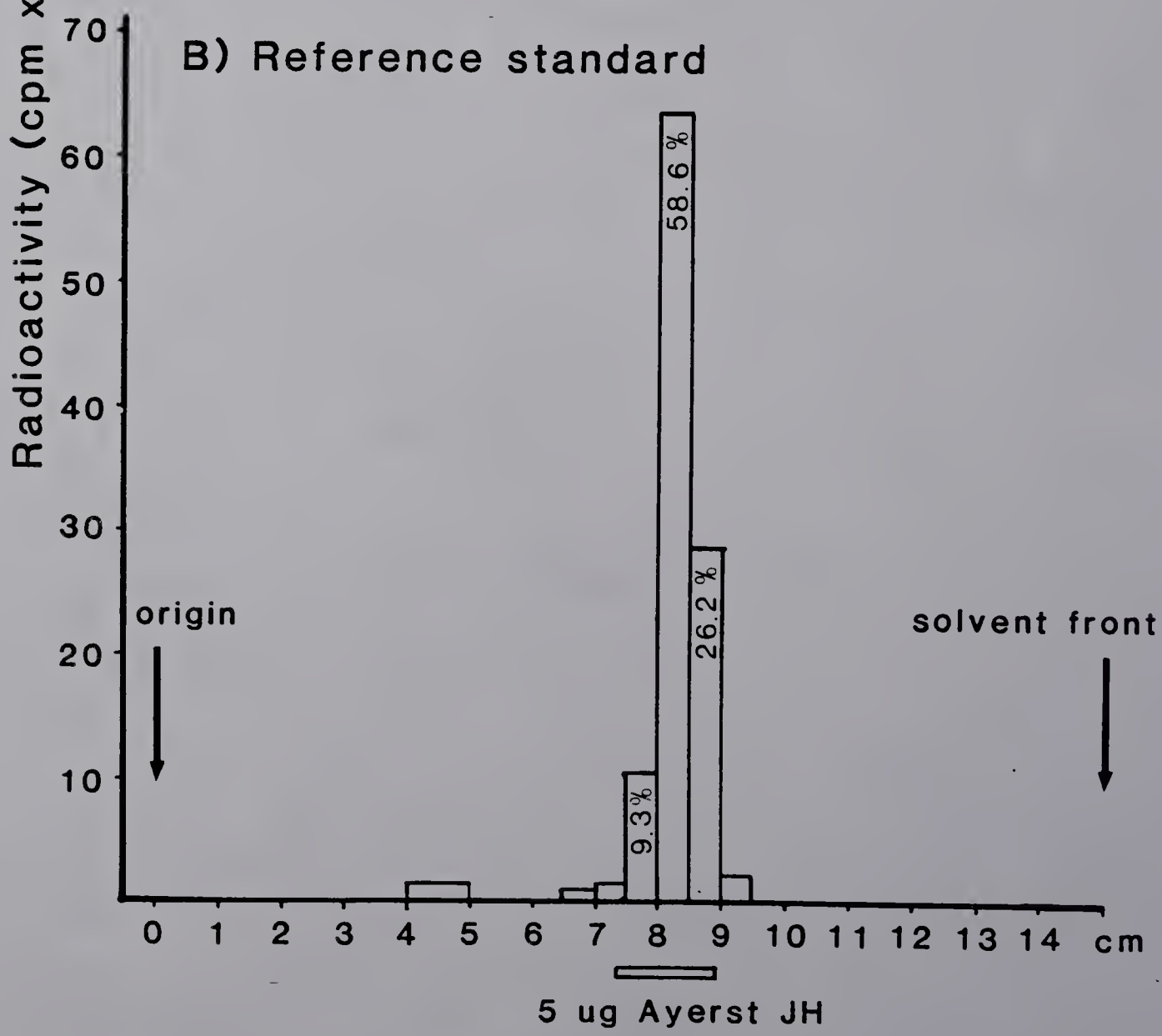
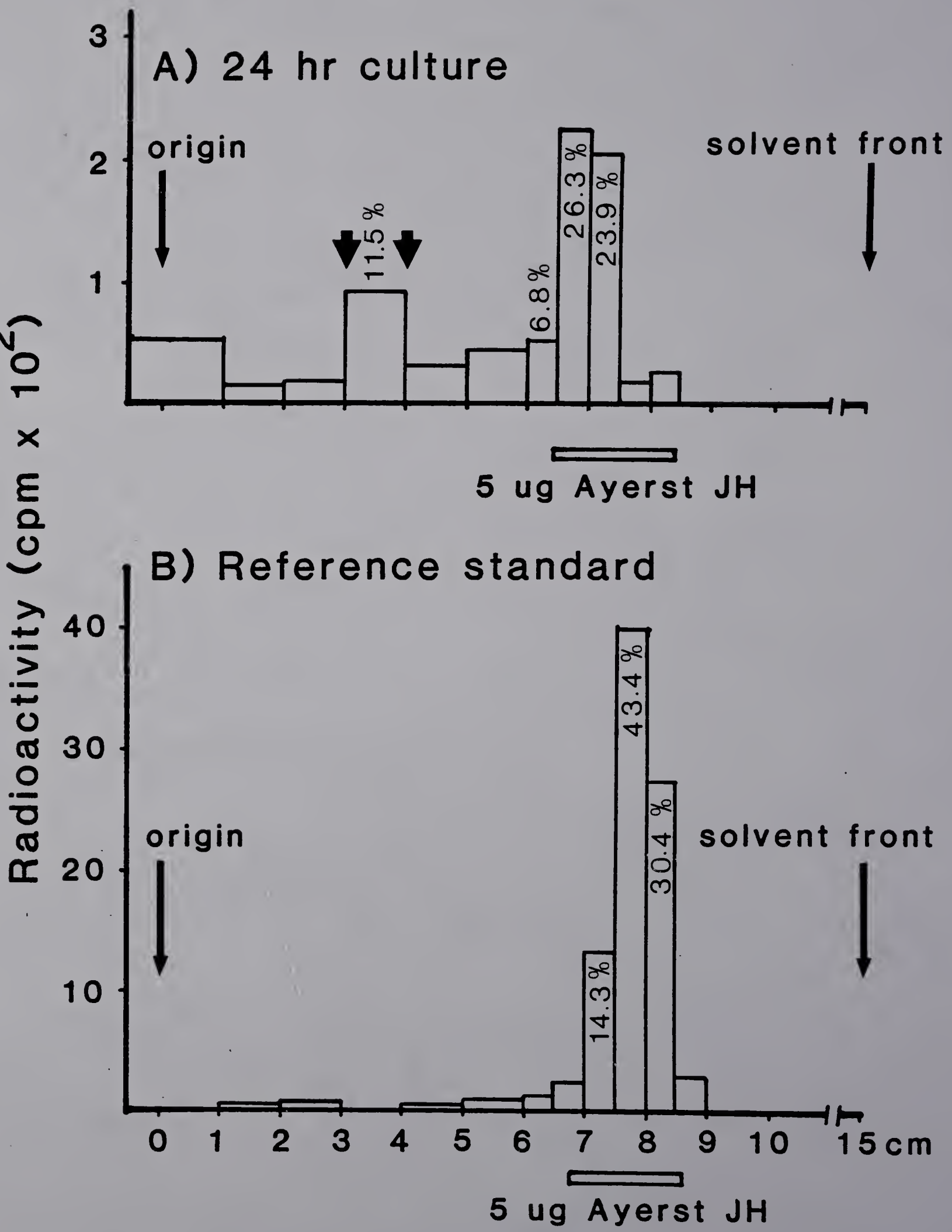

Radioactivity (cpm x 10²)
B) Reference standard
70
60
50
40
30
20
10
origin
solvent front
9.3%
58.6 %
26.2 %
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 cm
5 ug Ayerst JH

Figure 6. The distribution of radioactivity from the extract of CC-CA cultured for 24 hrs (A), and reference standard (B) on TLC normal phase developed in hexane/ethyl acetate 1:1.

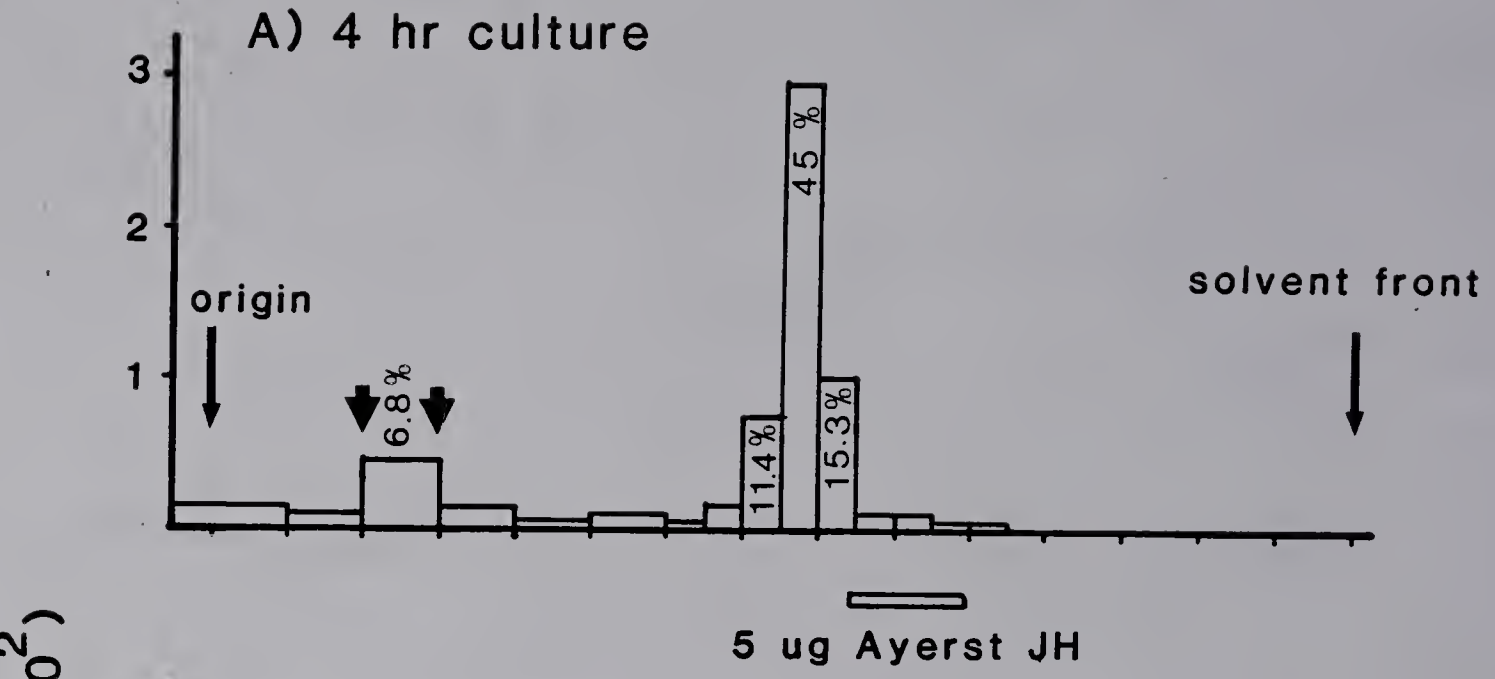

A) 24 hr culture
origin
solvent front
11.5 %
6.8 %
26.3 %
23.9 %
5 ug Ayerst JH
B) Reference standard
origin
solvent front
14.3 %
43.4 %
30.4 %
5 ug Ayerst JH
Radioactivity (cpm x 10²)
0 1 2 3 4 5 6 7 8 9 10 15 cm

Results presented in Figure 5 and Figure 6 revealed that some radioactivity (indicated with arrows) could be observed in an area with a mobility much less than the major radioactive region. Better separations of radioactivity in cultured samples from that of the reference standard was achieved when the solvent system was changed to hexane/ethyl acetate 2:1 (Figures 7-9). Likewise, standard [^{3}H]JH III always co-eluted with Ayerst JH I spot, whereas samples from gland cultures showed a 1.5 cm lag represented by the radioactivity. A small amount of radioactivity (indicated with arrows) also could be observed in an area behind the major radioactive regions (Figures 7-9). Figure 10 presents a typical result obtained using pooled samples cultured for four hours but using hexane/ethyl acetate 1:4 as the developing solvent. Under this condition the separation between sample and standard decreased.

TLC (reverse phase) separation and identification

Results of a SiC-18 reverse phase developed with 70% methanol are shown in Figures 11 (A)-(D). Figure 11-(A) shows the radioactivity distribution of the reference standard. Figure 11-(B) & (C) show the results when two CC-CA complexes were cultured for 28 hours while Figure 11-(D) shows the radioactivity pattern of pooled extracts of four glands from a four hour incubation. The result from the reference standard (Figure 11-(A)) shows that radioactivity of [^{3}H]JH III migrated to the front of Ayerst JH I spot. Results from samples cultured for 28 hours (Figures 11-(B),(C)) show several regions with high radioactivity: one at the origin, one migrating to the front of cold Ayerst JH I spot, and

Figure 7. The distribution of radioactivity from the extract of CC-CA cultured for 4 hrs (A), and reference standard (B) on TLC normal phase developed in hexane/ethyl acetate 2:1.


A) 4 hr culture
origin
6.8%
11.4%
45%
15.3%
solvent front
5 ug Ayerst JH

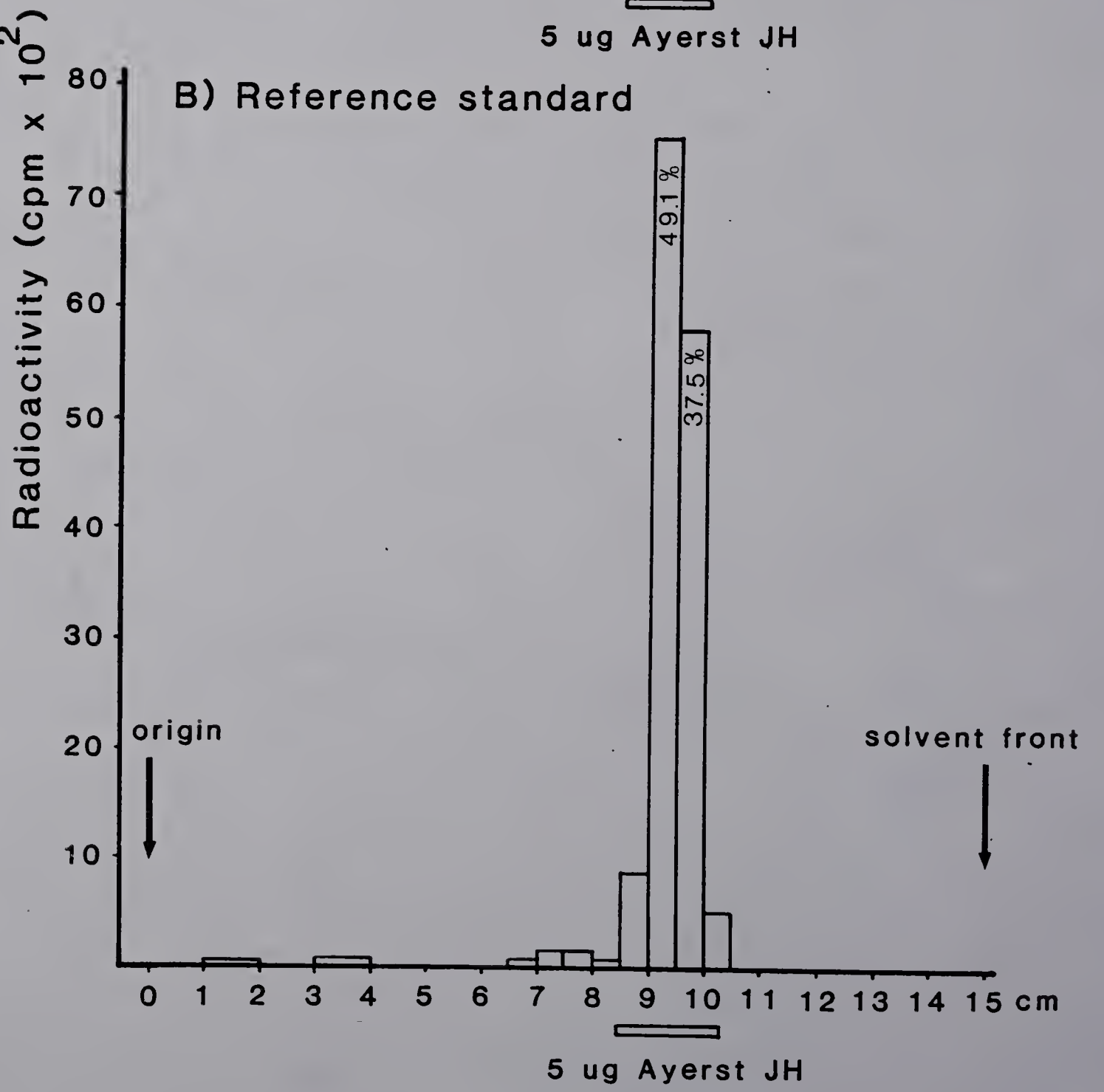

B) Reference standard
Radioactivity (cpm x 10²)
origin
49.1%
37.5%
solvent front
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 cm
5 ug Ayerst JH

Figure 8. The distribution of radioactivity from the extract of CC-CA cultured for 8 hrs (A), and reference standard (B) on TLC normal phase developed in hexane/ethyl acetate 2:1.

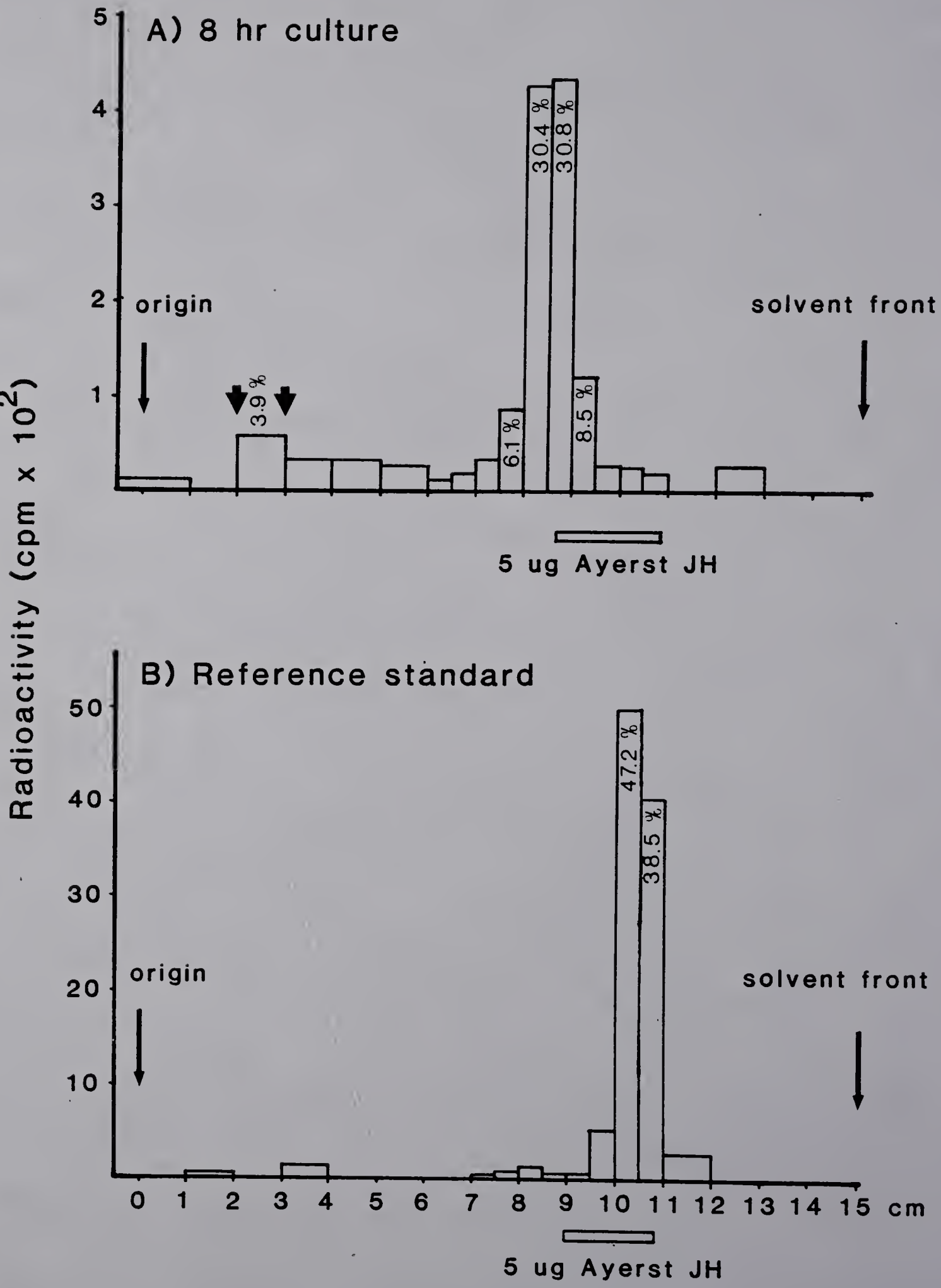

A) 8 hr culture
origin
solvent front
3.9 %
6.1 %
30.4 %
30.8 %
8.5 %
5 ug Ayerst JH
B) Reference standard
origin
solvent front
47.2 %
38.5 %
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 cm
5 ug Ayerst JH
Radioactivity (cpm x 10²)

Figure 9. The distribution of radioactivity from the extract of CC-CA cultured for 24 hrs (A), and reference standard (B) on TLC normal phase developed in hexane/ethyl acetate 2:1.

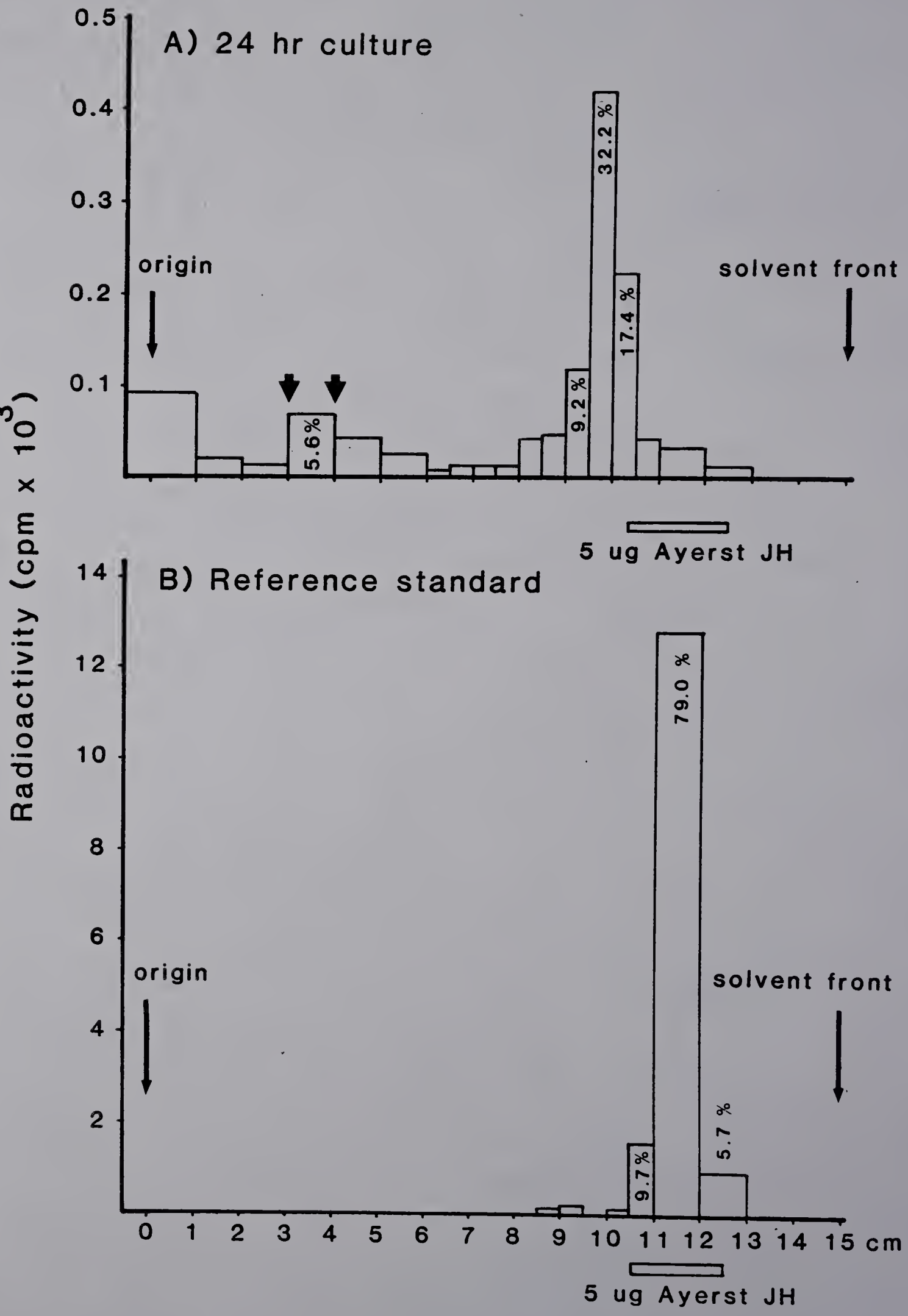
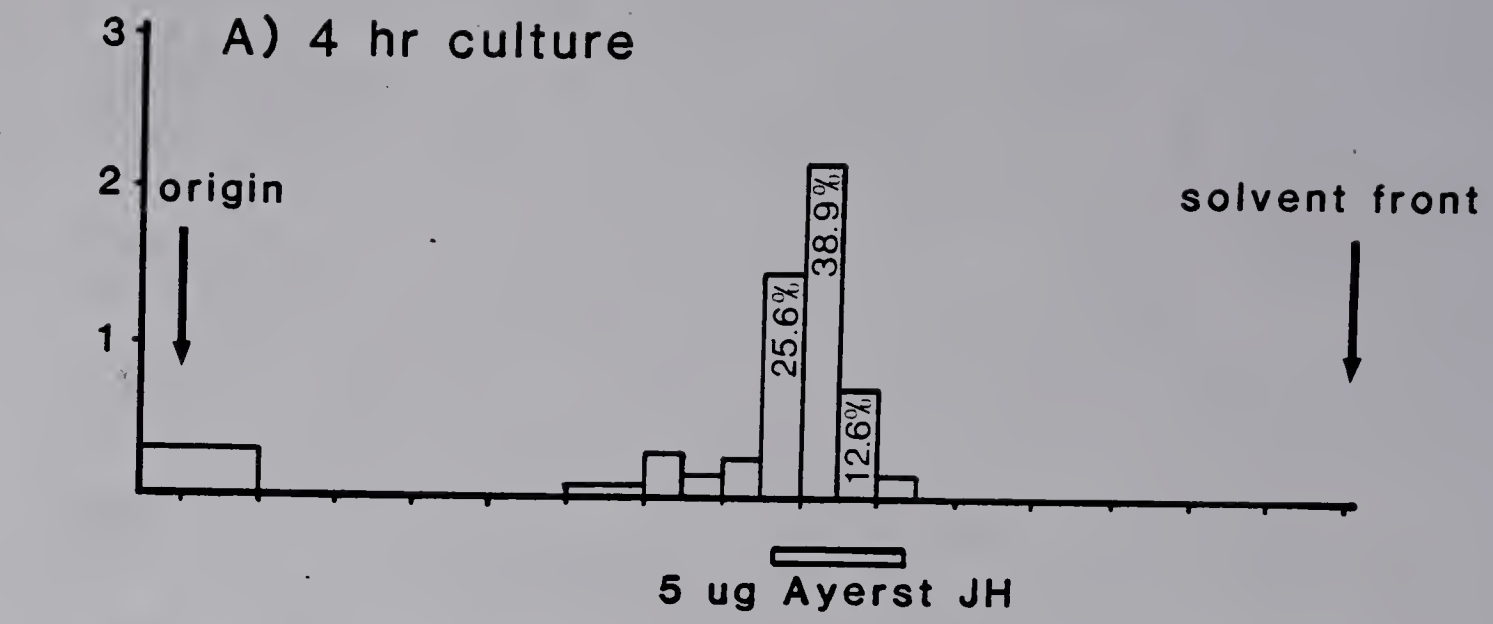

A) 24 hr culture
origin
solvent front
5.6%
9.2%
32.2%
17.4%
5 ug Ayerst JH
B) Reference standard
origin
solvent front
9.7%
79.0 %
5.7 %
5 ug Ayerst JH
Radioactivity (cpm x 10^3)
0.5
0.4
0.3
0.2
0.1
14
12
10
8
6
4
2
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 cm

Figure 10. The distribution of radioactivity from the extract of CC-CA cultured for 4 hrs (A), and reference standard (B) on TLC normal phase developed in hexane/ethyl acetate 1:4.


A) 4 hr culture
origin
solvent front
25.6%
38.9%
12.6%
5 ug Ayerst JH

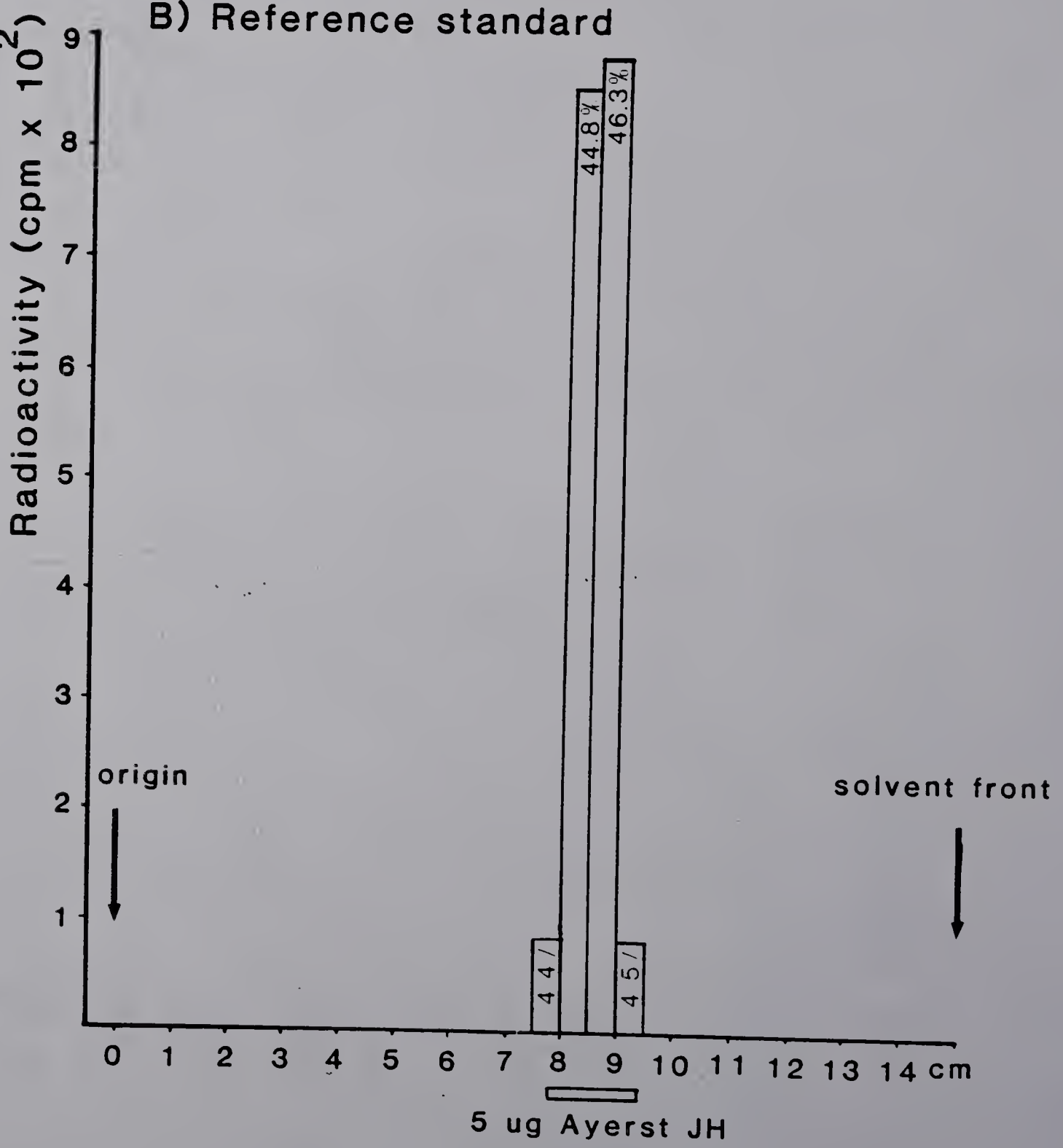
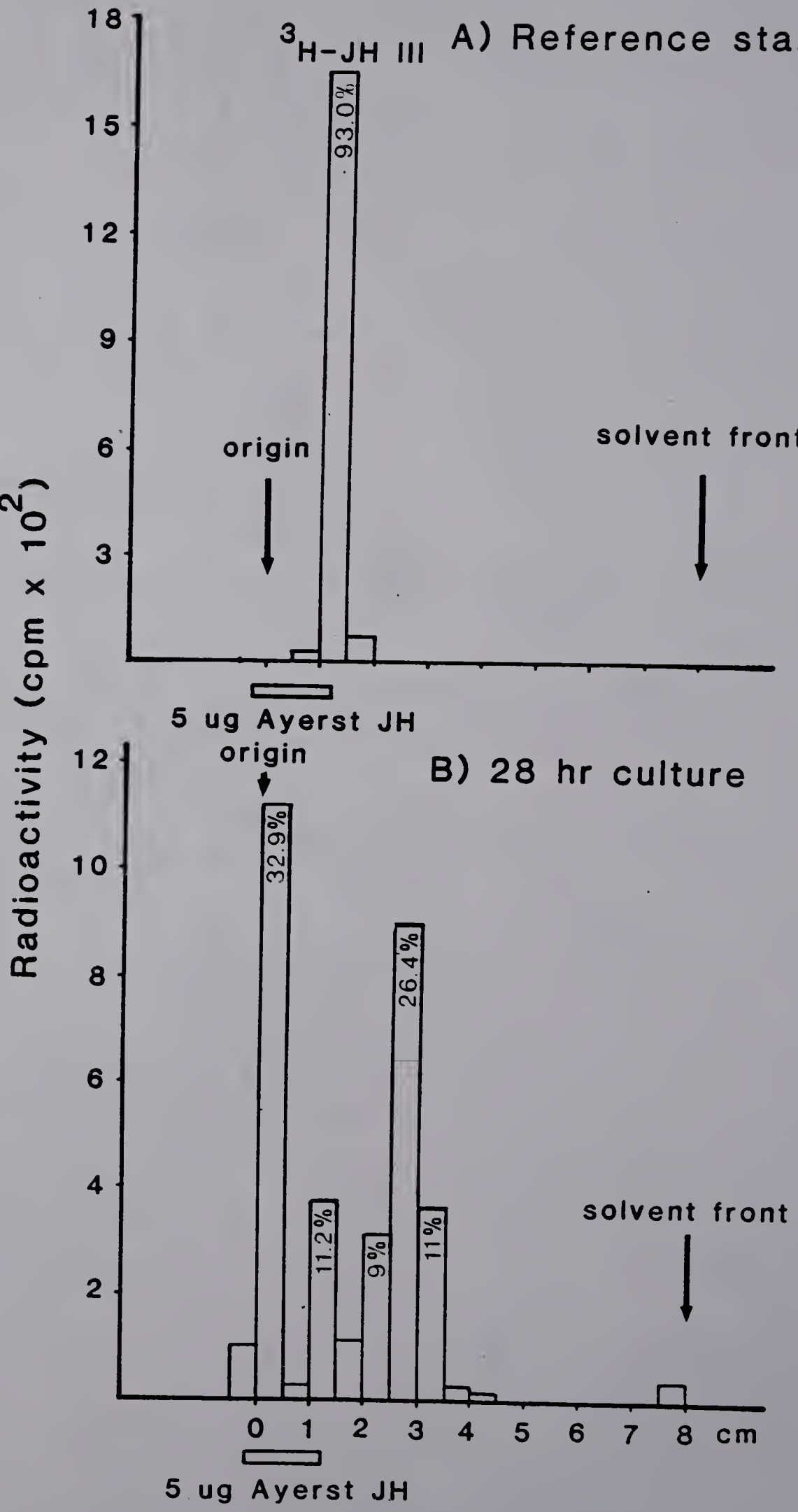
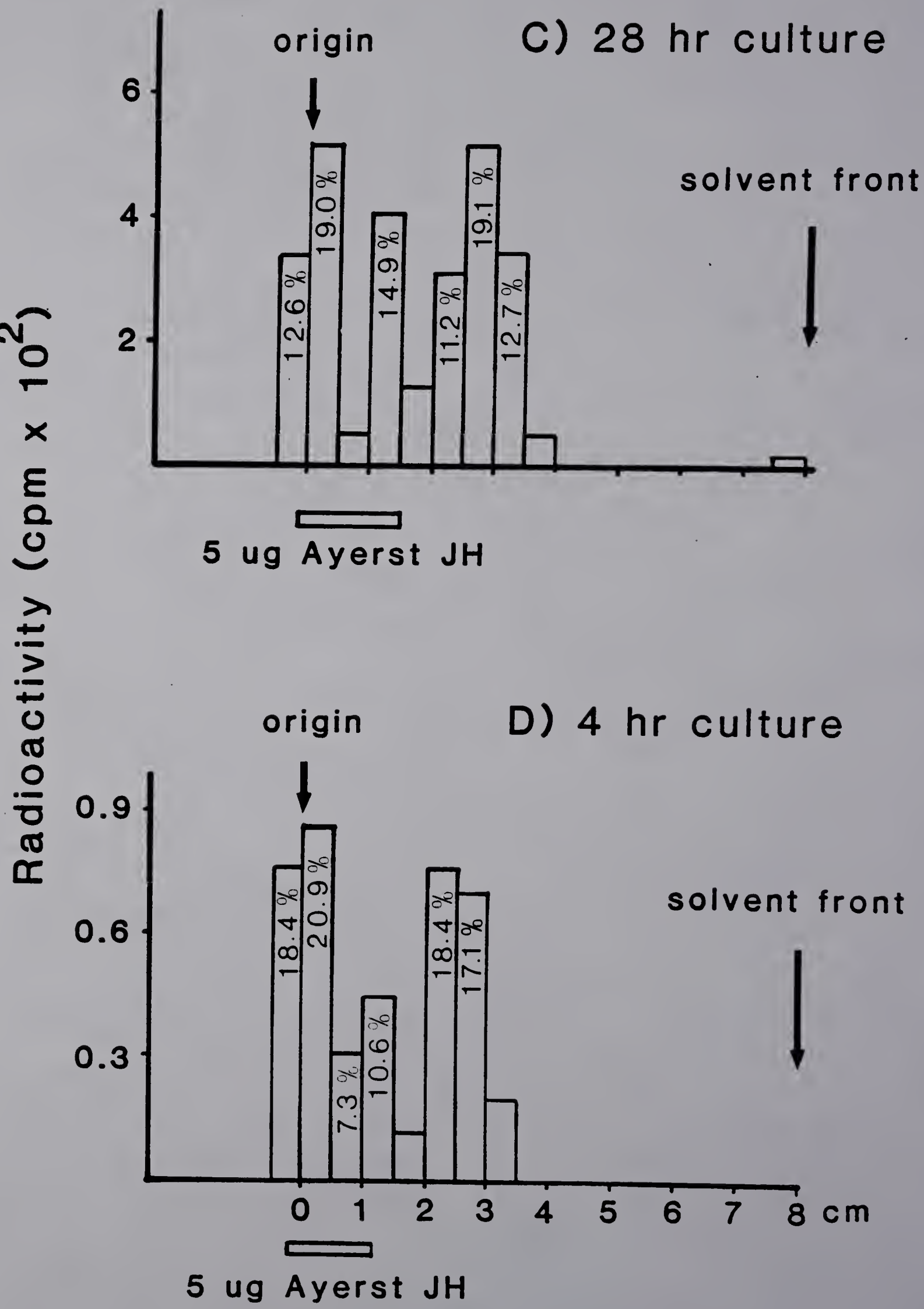

B) Reference standard
Radioactivity (cpm x 10²)
origin
solvent front
44.8%
46.3%
447
451
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 cm
5 ug Ayerst JH

Figure 11. The distribution of radioactivity of reverse phase SiC-18 TLC developed in 70% methanol: (A) reference standard, (B) & (C) extract from CC-CA cultured for 28 hrs, (D) extract from CC-CA cultured for 4 hrs.


A) Reference standard
^{3}H-JH III
93.0%
origin
solvent front
18
15
12
9
6
3
B) 28 hr culture
5 ug Ayerst JH
origin
32.9%
11.2%
9%
26.4%
11%
solvent front
12
10
8
6
4
2
0 1 2 3 4 5 6 7 8 cm
5 ug Ayerst JH
Radioactivity (cpm x 10^2)


C) 28 hr culture
origin
solvent front
6
4
2
12.6 %
19.0 %
14.9 %
11.2 %
19.1 %
12.7 %
5 ug Ayerst JH
D) 4 hr culture
origin
solvent front
0.9
0.6
0.3
18.4 %
20.9 %
7.3 %
10.6 %
18.4 %
17.1 %
0 1 2 3 4 5 6 7 8 cm
5 ug Ayerst JH
Radioactivity (cpm x 10^2)

a third one in an area 1.5 cm preceding the cold Ayerst JH I zone. Figure 11-(D) shows the result from samples cultured for four hours. Here three radioactive regions present: one at the origin, the second anterior to the Ayerst JH I spot, and the third zone 1.5 cm away from the spot of cold Ayerst JH I.

HPLC separation and identification

Results of reverse phase HPLC, using standard JH I and JH III, revealed that JH III had a retention time of 5 minutes while JH I a retention time at 7 minutes (Figure 12) under the conditions used in this study (see Materials and Methods). Reverse phase HPLC of standard [^{3}H]JH III also showed a 5 minute retention time (Figure 13). The corresponding radioactivity distribution of fractions collected for standard [^{3}H]JH III is shown in Figure 13. Most radioactivity could be detected ranging from 5-7 minutes with a peak at 5.5-6 minutes. There was a 0.75-1 minute difference between the detected absorbance peak and the peak of radioactive effluent. This time differential reflected that spent by the effluent passing from the UV-detector to the fraction collector.

Reverse phase HPLC of an extraction control (medium cultured without CC-CA) showed no peaks (Figure 14) in the regions where the standard JH III and JH I appeared (Figure 12). Results of reverse phase HPLC, as well as correlative radioactivity distributions from organ cultures of CC-CA complexes cultured under various conditions, provided the convincing result that radioactivity never coincided with the UV

Figure 12. Reverse phase HPLC of cold standard JH III (2 ng) and JH I (80 ng), absorbance 0.02 at 220 nm, 80% methanol with 1.2 ml/min flow rate, temperature 28°C, and chart rate 1 cm/min.

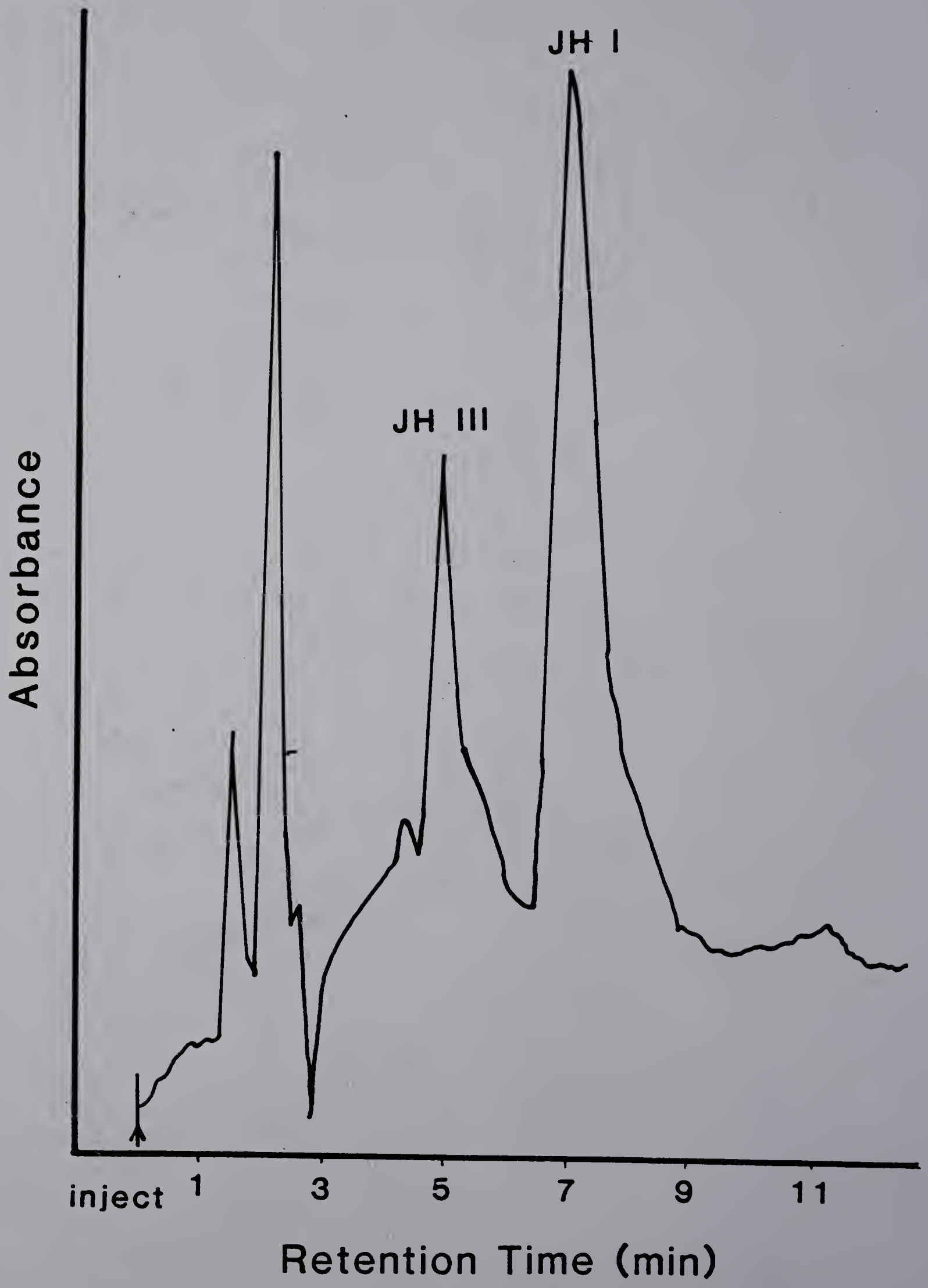

JH I
JH III
Absorbance
inject
1
3
5
7
9
11
Retention Time (min)

Figure 13. Reverse phase HPLC of standard [^{3}H]JH III (6.14 ng) accompanied with correlative radioactivity distribution of fractions collected, absorbance 0.02 at 220 nm, 80% methanol with 1.2 ml/min flow rate, temperature 28^{o}C, and chart rate 1 cm/min.

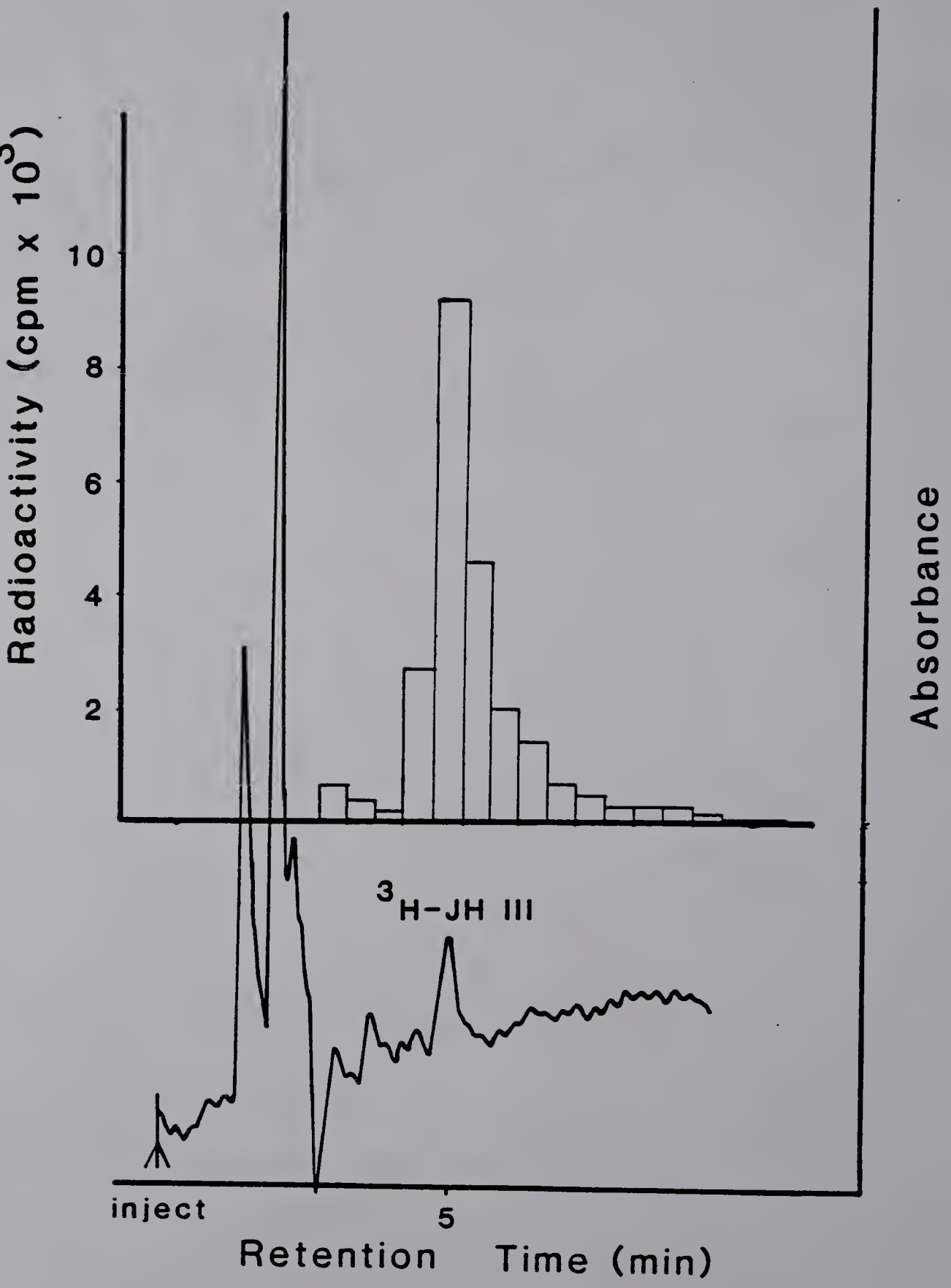
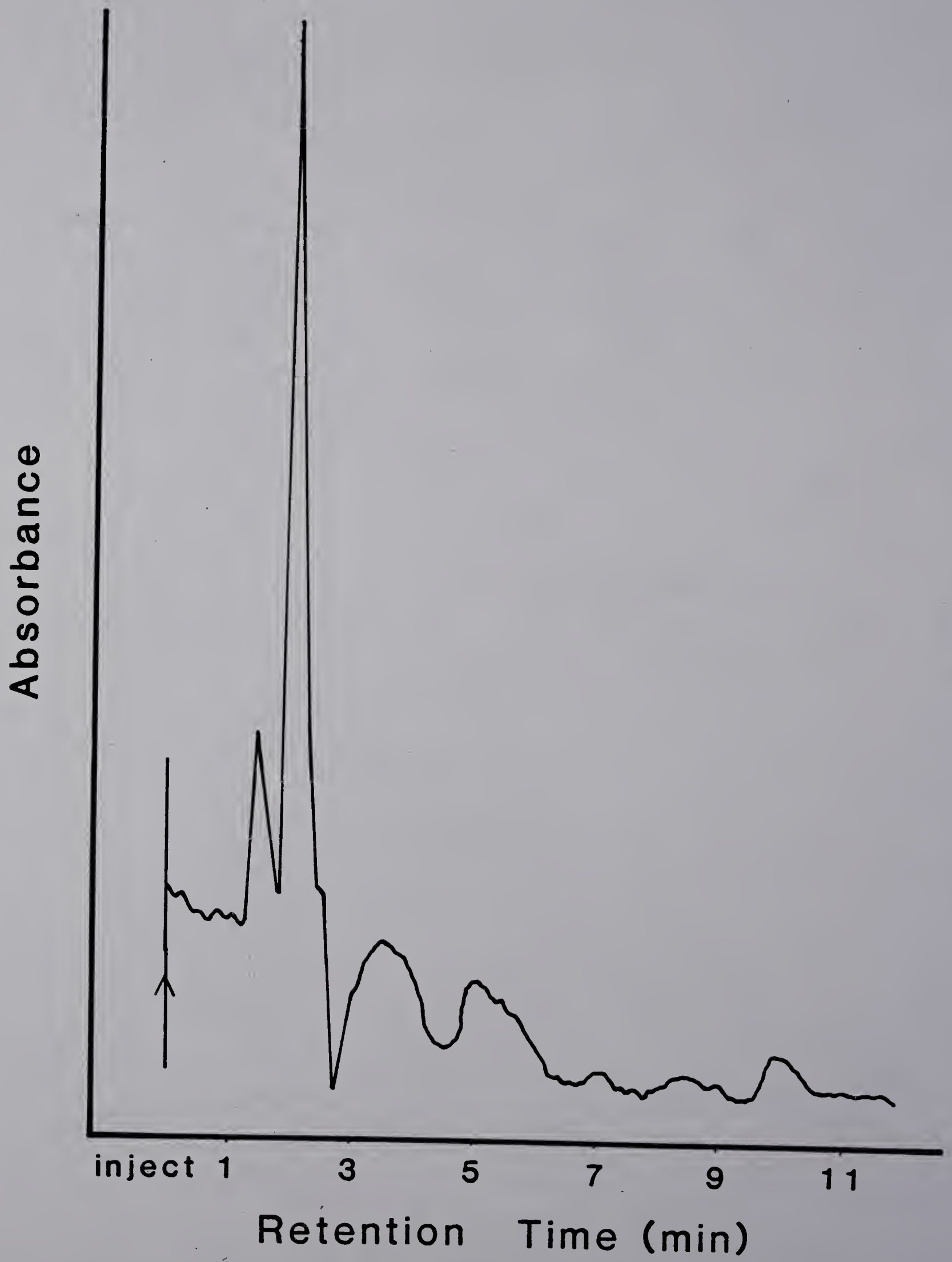

Radioactivity (cpm x 10^3)
10
8
6
4
2
Absorbance
^{3}H-JH III
inject
5
Retention Time (min)

Figure 14. Reverse phase HPLC of extraction control, absorbance 0.02 at 220 nm, 80% methanol with 1.2 ml/min flow rate, temperature 28°C, and chart rate 1 cm/min.


Absorbance
inject
1
3
5
7
9
11
Retention Time (min)

absorbing peaks which co-eluted with standard JH I and JH III (Figures 15-17). Almost all the radioactivity eluted before 5 minutes with the highest radioactivity peak at 3-4 minutes. Considering the time difference between the absorbance and radioactivity peaks, the effluent from radioactive CC-CA cultures should be detected by the UV detector at 2.5-3.5 minutes.

The results with ten glands (two glands per culture) incubated for four hours is presented in Figure 15. Compared with the standard cold JH I, JH III (Figure 12) and standard [^{3}H]JH III (Figure 13), the result in Figure 15 revealed that two non-radioactive peaks co-eluted with standard JH III and JH I respectively, and another peak between 2.6-2.8 minutes. Results of pooled biosynthesized products from organ cultures of six CC-CA complexes (two glands per culture) incubated for 24 hours and 48 hours (four glands cultured for 24 hours while two glands were cultured for 48 hours) are presented in Figure 16. Likewise, there were two non-radioactive peaks which co-eluted with the standard JH III and JH I respectively, and one conspicuous peak with radioactivity which appeared at 2.9 minutes. Results with the pooled iso-octane extractable products of four CC-CA complexes (two complexes per culture) incubated for four hours with medium pH of 7.5 are presented in Figure 17. There were also two non-radioactive peaks which co-eluted with standard JH III and JH I, and one peak at 2.8 minutes which corresponded to the peak of radioactivity of the collected fractions.

Effect of exogenous source of methionine on CC-CA activity

Reverse phase HPLC of the pooled iso-octane extractable products

Figure 15. Reverse phase HPLC of extract from 10 CC-CA cultured for 4 hrs and its correlative radioactivity distribution of fractions collected. Conditions of HPLC are described in Materials and Methods. Arrows indicate peaks with retention times at 2.6-2.8, 5, and 7 minutes.

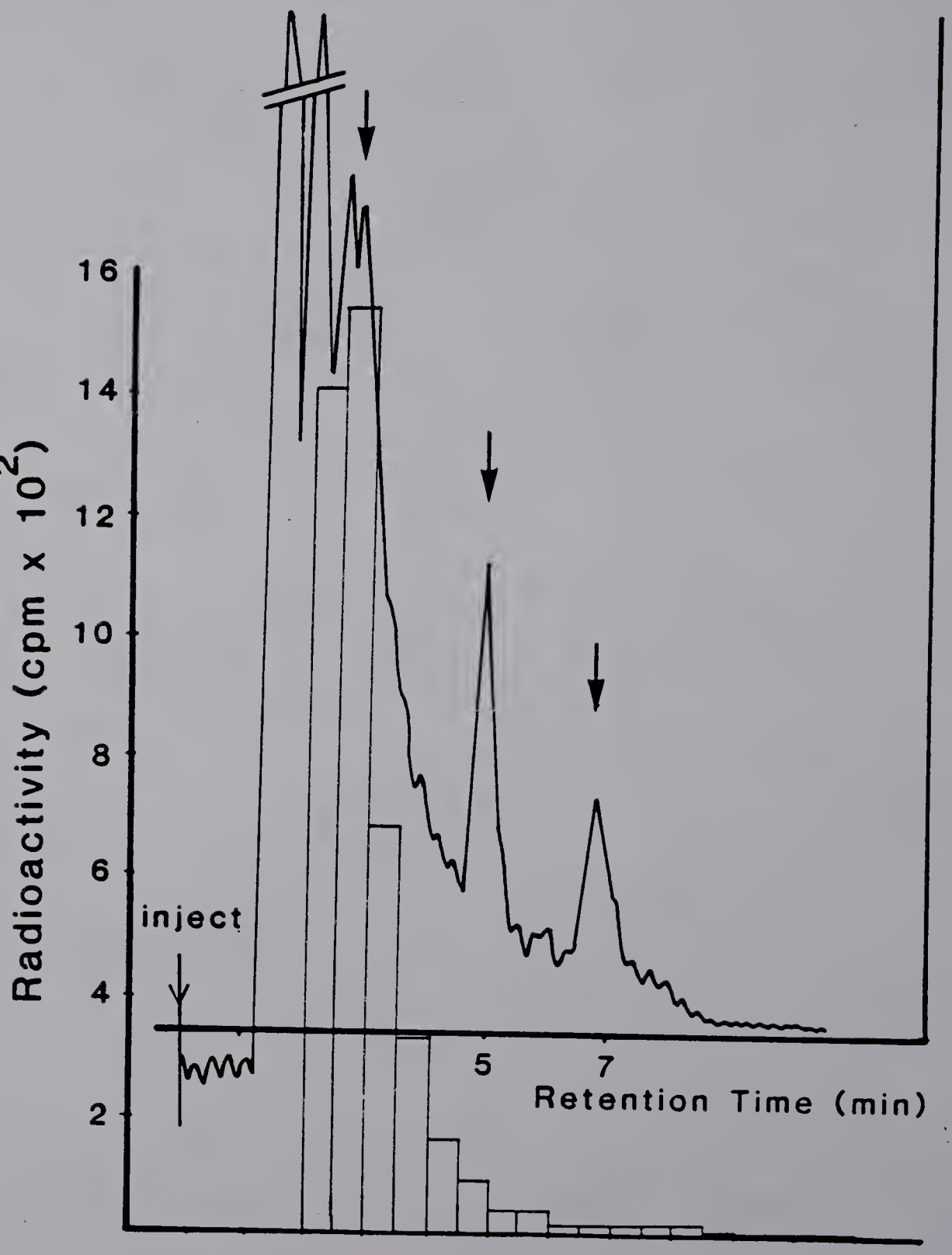

Radioactivity (cpm x 10²)
16
14
12
10
8
6
4
2
inject
5
7
Retention Time (min)
Absorbance

Figure 16. Reverse phase HPLC of extract from 6 CC-CA cultured for 24 or 48 hours and its correlative radioactivity distribution of fractions collected. Conditions of HPLC are described in Materials and Methods. Arrows indicate peaks with retention times at 2.9, 5, and 7 minutes.

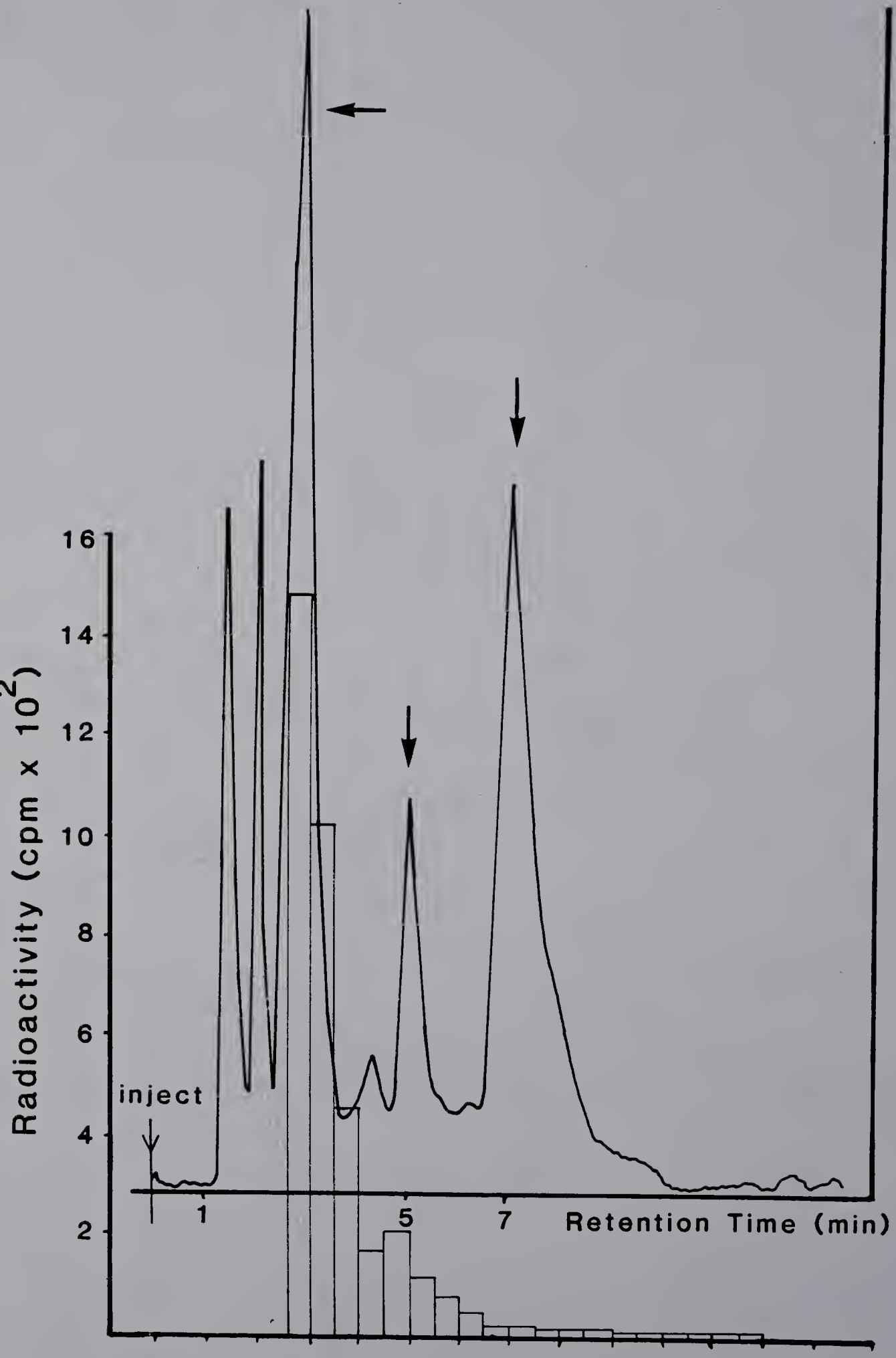

Radioactivity (cpm x 10^2)
16
14
12
10
8
6
4
2
inject
1
5
7
Retention Time (min)
Absorbance

Figure 17. Reverse phase HPLC of extract from 4 CC-CA cultured for 4 hrs at pH 7.5 and its correlative radioactivity distribution of fractions collected. Conditions of HPLC are described in Materials and Methods. Arrows indicate peaks with retention times at 2.8, 5, and 7 minutes

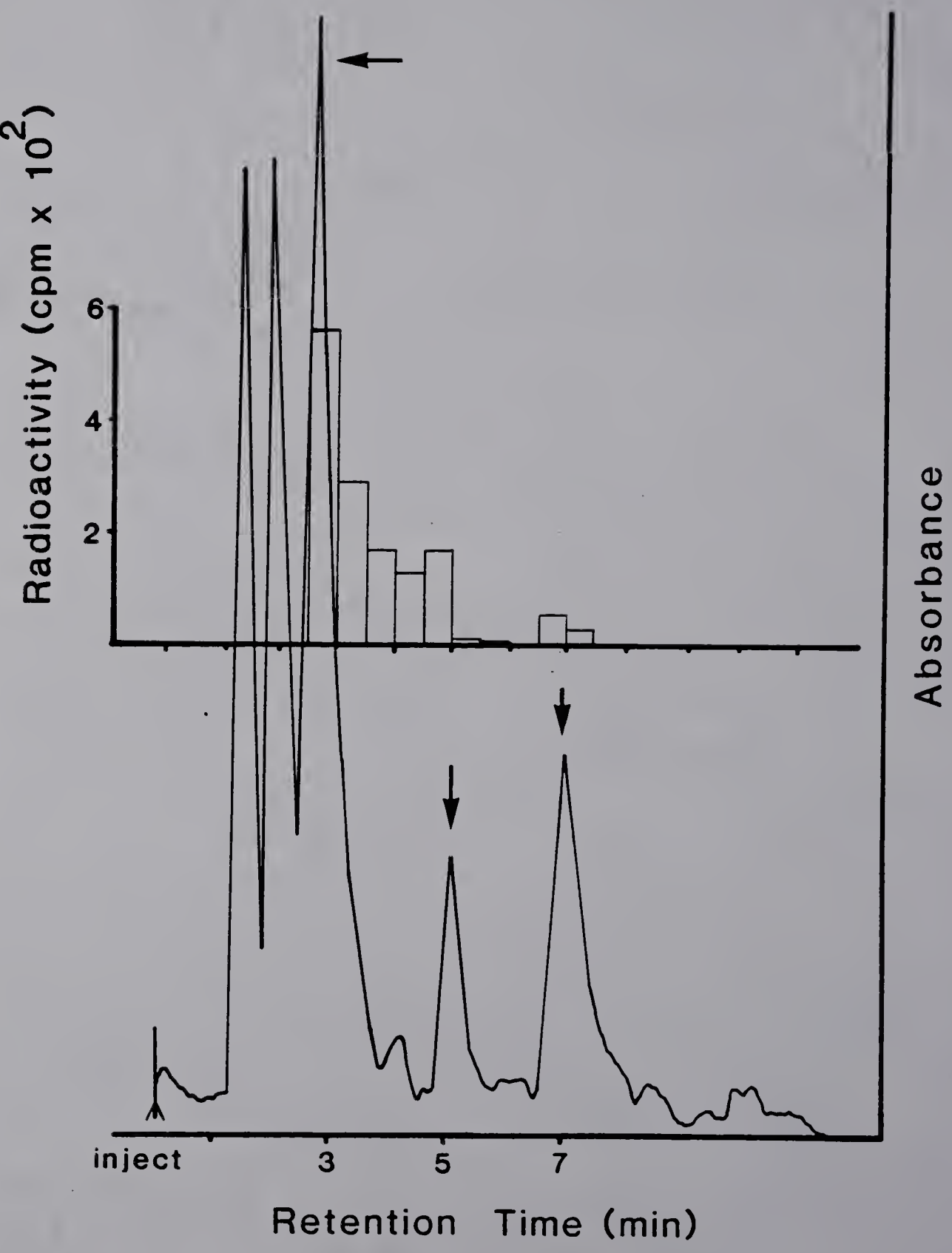
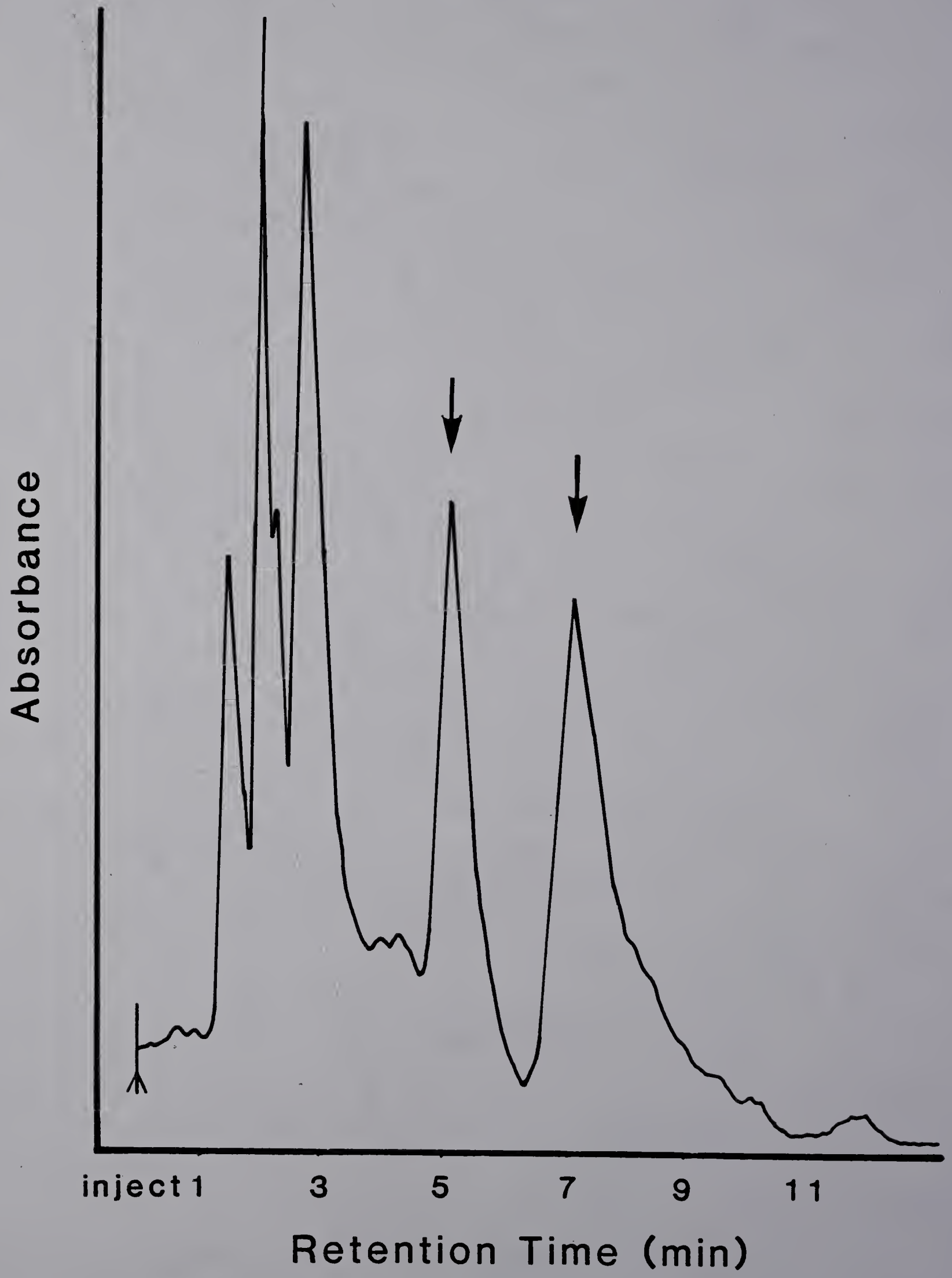

Radioactivity (cpm x 10^2)
6
4
2
Absorbance
inject
3
5
7
Retention Time (min)

Figure 18. Reverse phase HPLC of extract from 8 CC-CA cultured in medium without exogenous methionine for 4 hours. Conditions of HPLC are described in Materials and Methods. Arrows indicate peaks with retention times at 5 and 7 minutes.


Absorbance
inject
1
3
5
7
9
11
Retention Time (min)

(Figure 18) from eight CC-CA complexes (two complexes per culture) incubated in medium without methionine for four hours revealed two peaks that co-eluted in complete coincidence with the unlabelled standards of JH III and JH I, and one peak shown at 2.8 minutes. The result of this experiment was similar to those where glands were cultured with exogenous methionine.

Discussion and Conclusions

Judy et al., (1973a; 1973b; 1975), Schooley et al., (1973), and Jennings et al., (1975) employed long-term in vitro culture techniques and chromatographic analyses including thin layer chromatography (TLC), gas-liquid chromatography (GLC), high resolution liquid chromatography (HRLC) for the purification and identification of JH biosynthesized by the CA from various species. Roller and Dahm (1970), and Muller et al., (1974) further confirmed the structure of biosynthesized products from cultured CA using gas chromatography - mass spectrometry (GC-MS). Later, researchers applied short-term CA culture technique with chromatographic analyses and scintillation spectrometry to study JH biosynthesized by CA from many species (Pratt and Tobe, 1974; Tobe and Pratt, 1974a). However, no GC-MS analysis was employed to identify the biosynthesized radioactive products.

To develop an in vitro culturing technique for estimating JH biosynthesis by CA from P. regina, two important objectives were: (a) to develop an in vitro culture system which provided the highest

incorporation of [^{3}H]methionine, and (b) to confirm that the radiolabelled product is JH.

Development of methods

In this study, cultured CC-CA were taken from females 24 hours after liver feeding. This period was selected based on the studies of Orr (1964a) and Stoffolano (1974) which indicated that the CA should be activated within 2 days after feeding on a protein source. It is possible that highly active CA could be found several hours after liver feeding since the protein meal somehow functions as a "trigger" for CA activation (Orr, 1964a; Pappas and Fraenkel, 1977; 1978; Fraenkel and Hollowell,1979), and results of ovaries examined in this study revealed that over 97% of liver fed flies already had deposited yolk 24 hours after liver feeding. Control flies (maintained on a sugar and water diet) were prevented from having access to any exogenous protein sources by rearing fewer females singly and in clean cages because ovarian development has been shown to be initiated by feeding on dead flies and excreta (Pappas and Fraenkel, 1977). Observations on ovarian development indicated that none of the ovaries of control flies developed beyond stage 2. This result agrees with those reported by Pappas and Fraenkel (1977) and Stoffolano (1974). However, the ovarian development of liver fed flies showed some variations which might partly be attributed to innate differences among flies. Another possibility is that insufficient protein was ingested by some individuals because Orr (1964a) pointed out that a specific amount of dietary protein is needed for the activation of the CA. The variations observed on ovarian

development might also reflect those observed on the incorporation rates of methionine by CA from individual flies. Therefore, future studies involving CA donor insects should include a careful examination of the reproductive state of the females. This will allow researchers to better explain the variations observed in their studies.

The reason that the CC-CA complex, instead of CA alone, was used for all culturing experiments was to minimize any possible injury to the CA. Other researchers cultured either Br-CC-CA (Roller and Dahm, 1970; Jennings et al., 1975) or CC-CA together (Judy et al., 1973a; Muller et al., 1974; Tobe and Stay, 1977; Kramer, 1978; Weaver et al., 1980; Khan et al., 1982; Feldlaufer et al., 1982; Bowers et al., 1983) while some others cultured the CA alone (Judy et al., 1973b; most of Tobe and Pratt and their co-researchers' work; Lanzrein et al., 1978; Roseler and Roseler, 1978; Roseler et al., 1980; de Kort et al., 1981; Granger et al., 1982). Judy et al., (1973a; 1975) reported that there was no effect on hormone production by CA of culturing Br-CC-CA, CC-CA, CA alone, or of adding fat body into the culture medium. Ferenz and Kaufner (1981) indicated that addition of an isolated CC or incubation of a CC-CA complex did not change the JH synthesis pattern, however, sometimes the CC can affect the CA and somehow influence it to remain active longer, or it can elevate rates of JH synthesis by CA of Locusta migratoria. Tobe and Pratt (1975a) raised the question of whether a neural connection to the brain or the presence of circulating neurosecretion are necessary for either the induction or maintenance of highs levels of CA activity in vitro and concluded that a CA deprived of

its nervous connections and suspended in a medium containing no humoral trophic factors may lose its activity after a period of several hours. Couillaud et al., (1984) reported that severance of nervi corporis allati I in one day old female Locusta migratoria results in a significant decrease of JH biosynthesis by the CA. Therefore, CC-CA were dissected and cultured together in this study.

The 91% of standard JH recovery was obtained by using iso-octane and previously described procedures to extract JH from medium in this study. Good recovery of C_{16}JH with iso-octane was also reported by Feyereisen and Tobe (1981).

The result of the extraction source experiment provided evidence that products synthesized by cultured CC-CA were released into the surrounding medium as soon as they were synthesized. This had previously been demonstrated with other insects (Pratt and Tobe, 1974; Tobe and Pratt, 1974a; 1974b; Tobe and Stay, 1977; Roseler and Roseler, 1978; Weaver et al., 1980).

Buck (1953) indicated that there is a close correlation between normal diet and hemolymph Na^+/K^+ ratio in insects and reported that carnivores have ratios greater than 1. de Loof (1982) also mentioned that having a high Na^+ and low K^+ concentration in hemolymph is one of the typical characteristics of Diptera. Although there is no information on the hemolymph Na^+/K^+ ratio of P. regina, comparison of the Na^+/K^+ ratio of Phormia saline (Chen and Friedman, 1975) and of the MEM (Eagle, 1959) used as culture medium revealed an ratio of greater than 1, which suggested the physiological suitability of MEM for P. regina. This finding coincided with the suggestion on the selection of

a culture medium which was proposed by Weaver et al., (1980) for T. molitor.

L-methionine concentration. Previous studies indicated methionine concentration in the culturing medium is important. Tobe and Pratt (1974a) investigated the influence of incubation conditions on the rate of JH biosynthesis by CA of S. gregaria and found the optimum L-methionine concentration ranging from 0.1 to 0.4 mM which encompasses the physiological concentration of this substrate in the hemolymph. Weaver et al (1980) reported that the high rates of hormone production by CA of T. molitor were obtained at 0.3-0.9 mM with the optimum concentration at 0.3-0.4 mM. In contrast, with P. regina the maximum incorporation of radiolabelled methionine occurred when the total methionine concentration was 0.05 mM (Table 6).

Medium pH. Although the highest incorporation rate by CA was obtained at pH 7.5, pH 7.0 was employed as the pH value of the culture medium in all experiments performed in this study due to the hemolymph pH value of 7.08 in P. regina (Chap. II). Similar results on S. gregaria were reported by Tobe and Pratt (1974a). They found that even though glands had a maximum synthetic activity at pH 8.0, their activity was more reproducible in the physiological range of pH 7.0-7.5. Kramer (1978) observed that the synthetic activity of the CA was constant between pH 6 and 7.5 based on the study with L. decemlineata. Weaver et al., (1980) reported that the highest rates of hormone biosynthesis by CA from T. molitor were achieved over a pH range of 7.0-7.4. All these results indicate that the physiological suitable pH of the culture

medium is extremely important for *in vitro* cultures.

Incubation duration. CA activity of *P. regina* in terms of unit production (presented as pmol x gland^{-1} x hr^{-1}) could remain constant for 24 hours, while the highest rate was observed with 4 hour incubation period in this study. In contrast, Tobe and Pratt (1974a) reported that the rate of incorporation was constant for up to 4 hours in *S. gregaria*, thereafter declining (Pratt *et al.*, 1975). Lanzrein *et al.*, (1978) demonstrated that JH synthetic rate by CA of *N. cinerea* was linear over a period of 2-8 hours and then remained constant during the period from 8 to 24 hours. Weaver *et al.*, (1980) observed that hormone release from CA of *T. molitor* was essentially constant for 2-3 hours and diminished progressively. Ferenz and Kaufner (1981) reported that CA of *L. migratoria* rapidly lost their activity after 2 or 3 hours of incubation. The rate of incorporation of [^{14}C-methyl]methionine into JH by CA of *L. decemlineata* was linear over a period of 6 hours (de Kort *et al.*, 1981). Furthermore, Weaver *et al.*, (1980) suggested that only short-term assays can provide true estimates of glandular activity which reflect rates of hormone production by the CA immediately prior to their extirpation.

Chemical confirmation

Using various chromatographic analyses, this study provids convincing evidence that the synthesized radiolabelled products from CC-CA preparations of *P. regina* under *in vitro* were not either JH III or JH I but something also soluble in iso-octane. This result is different from that reported on *Diploptera punctata* by Feyereisen and Tobe (1981), who reported that *D. punctata* CA do not release any other iso-octane-

soluble metabolite of methionine into the culture medium except C_{16}JH. Chromatographic data revealed that these unknown radioactive products are more polar than JH III. In this study, it is possible that these unknown radiolabelled products might be products from the CC and hypocerebral ganglion since CC-CA were cultured together in our experiments. However, further identification of the products synthesized *in vitro* by CC-CA from *P. regina* must be made before any conclusions are possible.

Pratt and Tobe (1974) used [^{3}H]farnesenic acid and [^{14}C]methionine as precursors and somehow noted that there was some ^{3}H-radioactivity observed in areas of the chromatograph containing compounds of greater polarity than any know JH. Bowers *et al* (1983) also indicated incorporation of significant radioactivity into an additional area when [^{14}C]methionine was used as precursor. This unidentified radiolabelled product also had higher polarity than any known JH.

From normal phase TLC a small radioactive peak tended to appear from samples cultured for longer duration. It might imply some enzymatic degradations occurred within the system employed which might be important in terms of considering incubation duration and studying metabolism of these products. According to the results of reverse phase TLC, it is possible that several radioactive compounds are synthesized by CC-CA of *P. regina*.

Since HPLC provided a sensitive method for detecting JH at nanogram levels, it is very important to clean the injection port and syringe thoroughly before and after each injection to prevent any possible

contamination. Poor resolution of radioactivity distribution of effluent collected by HPLC in this study might be due to some degree of mixture occurring before collection.

Finally, from results of reverse phase HPLC, it is interesting to note that the *in vitro* cultured CC-CA can synthesize compounds giving 2 peaks which co-elute with standard JH III and JH I without incorporating any provided [methyl-^{3}H]methionine into those compounds (Figs. 15, 16, 17). This was not in agreement with the results of Pratt and Tobe (1974) and Judy *et al.*, (1973) who showed that the methyl group of methyl-labelled L-methionine is incorporated into JH biosynthesized by the CA *in vitro*. Experiments (Fig. 18) on incubating CC-CA in medium without any exogenous methionine provided evidence indicating that within the CC-CA there might be some other sources which can be utilized by the gland to synthesize compounds which co-elute with standard JH III and JH I, or there might be a methyl donor pool existing within CC-CA so that exogenous methionine is not necessary. This result also did not agree with that of Tobe and Pratt (1974a) and Weaver *et al.*, (1980). They indicated that there were no other potential methyl donors in the CA or medium for JH biosynthesis except L-methionine.

In Diptera, Girard *et al.*, (1976) identified JH I in whole body extracts of *M. domestica*. Klages *et al.*, (1980) reported that JH III was exclusively found in adult *Drosophila hyedi* while JH I also can be detected in traces in 3rd instar larvae. Baker *et al.*, (1983) reported that JH III was identified in whole-body extracts of larval and adult *Aedes aegypti*. Results of this *in vitro* study showed that CC-CA of adult *P. regina* can produce compounds which co-elute with JH III and JH

I. If further identification can be confirmed, it will be convincing evidence that both JH I and JH III are the JH of adult P. regina.

Using this in vitro technique, comparison of the incorporation of [^{3}H]methionine by active and inactive CC-CA demonstrated that a source of protein in the diet is essential for activation of this gland in P. regina. This idea had been previously investigated by Orr (1964a), Pappas and Fraenkel (1977; 1978), and Fraenkel and Hollowell (1979). It is now possible to monitor directly the CA activity of P. regina and determine its role during the reproductive cycle, also to investigate the regulation of JH biosynthesis by CA in P. regina by incorporating various hormonal and humoral factors into the culturing medium. Further research should include (a) identification of the radiolabelled unknown product using GC-MS analysis, and (b) development of an optimal culturing condition for JH biosynthesis by combining methods employed in this study. However, quantification of JH biosynthetic rates by estimating the incorporation rate of [^{3}H]methionine into JH by CA sould be reevaluated since the CA can utilize methyl-group donors from sources other than quantified exogenous methionine incorporated into culture medium. More reliable methods to quantify JH biosynthesis rates should be investigated.

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